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THE USE OF p57/KIP2 TO EVALUATE AGENTS FOR THE TREATMENT AND PREVENTION OF CANCER

The present invention is directed to a method of determining if cancer cells are resistant to an agent by determining the p57/KIP2 level in the cancer cells prior to contact with the agent; contacting the cancer cells with the agent; determining the p57/KIP2 level in the cancer cells after contact with the agent; comparing the p57/KIP2 level in the cancer cells after contact with the agent to the p57/KIP2 level in the cancer cells prior to contact with the agent; where an increase in the p57/KIP2 level in the cancer cells after contact with the agent compared to the p57/KIP2 level in the cancer cells prior to contact with the agent indicates the cancer cells are resistant to the agent.

The present invention is directed to a method of determining if cancer cells are resistant to an agent by contacting the cancer cells with the agent; determining the p57/KIP2 level in the cancer cells after contact with the agent; where an increase in the p57/KIP2 level in the cancer cells after contact with the agent indicates the cancer cells are resistant to the agent.

The present invention is directed to a method of determining if cancer cells are sensitive to an agent by determining the p57/KIP2 level in the cancer cells prior to contact with the agent; contacting the cancer cells with the agent; determining the p57/KIP2 level in the cancer cells after contact with the agent; comparing the p57/KIP2 level in the cancer cells after contact with the agent to the p57/KIP2 level in the cancer cells prior to contact with the agent; where no increase in the p57/KIP2 level in the cancer cells after contact with the agent compared to the p57/KIP2 levels in the cancer cells prior to contact with the agent indicates the cancer cells are sensitive to the agent.

The present invention is directed to a method of determining if cancer cells are sensitive to an agent by contacting the cancer cells with the agent; determining the p57/KIP2 level in the cancer cells after contact with the agent; where no increase in the p57/KIP2 level in the cancer cells after contact with the agent indicates the cancer cells are sensitive to the agent.

The present invention is directed to a method of identifying an agent effective for the treatment of a cancer by determining the p57/KIP2 level in cancer cells prior to contacting with the agent; contacting the cancer cells with the agent; determining the p57/KIP2 level in the cancer cells after contacting with the agent; comparing the p57/KIP2 level in the cancer cells after contacting with the agent to the p57/KIP2 level in the cancer cells prior to contacting with the agent; where no increase in the p57/KIP2 level in the cancer cells after contacting with the agent compared to the p57/KIP2 level in the cancer cells prior to contacting with the agent indicates the agent is effective for the treatment of a cancer.

The present invention is directed to a method of determining the therapeutic effectiveness of an agent by contacting normal cells with the agent; determining the p57/KIP2 level in the normal cells after contacting with the agent; contacting cancer cells with the agent; determining the p57/KIP2 level in the cancer cells after contacting with the agent; comparing the p57/KIP2 level in the normal cells after contacting with the agent to the p57/KIP2 level in the cancer cells after contacting with the agent; where a higher p57/KIP2 level in the normal cells compared to the p57/KIP2 level in the cancer cells indicates the agent is effective for the treatment of cancer.

The present invention is directed to a method of optimizing the formulation of an agent for the treatment of a cancer by contacting cancer cells with a first formulation of the agent;

determining the p57/KIP2 level in the cancer cells contacted with the first formulation of the agent; contacting cancer cells with a second formulation of the agent; determining the p57/KIP2 level in the cancer cells contacted with the second formulation of the agent; comparing the p57/KIP2 level in the cancer cells contacted with the first formulation of the agent to the p57/KIP2 level in the cancer cells contacted with the second formulation of the agent; where the formulation with the lower level of p57/KIP2 indicates the formulation of the agent more effective for the treatment of a cancer.

As used herein an agent includes any agent used for the treatment or prevention of cancer. For example, an agent includes, but is not limited to, chemical agents for the treatment or the prevention of cancer. Such chemical agents include, for example, the green tea polyphenols (GTPPs) such as (-)-Epicatechin (EC), (-)-Epigallocatechin (EGC), (-)-Epicatechin-3-gallate (ECG) or (-)-Epigallocatechin-3- gallate (EGCG).

As used herein, cancer cell includes any cancer cell in which an induction in the level of p57/KIP2 is observed. For example, this includes, but is not limited to, epithelial carcinoma cell lines, such as oral squamous carcinoma cell lines, metastatic oral carcinoma cell lines, or breast epithelial carcinoma cell lines, and human carcinomas of epithelial origin, such as oral squamous carcinoma, metastatic oral carcinoma or breast epithelial carcinoma.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

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Example 1

Chemopreventive Effects of Green Tea Polyphenols Correlate with Reversible Induction of p57 Expression

Green tea polyphenols are known to induce apoptosis in certain types of tumor cells. However, mechanism(s) that enables normal cells to evade the apoptotic effect is still not understood. In this study, Western analysis combined with cycloheximide treatment was used to examine the effects of green tea polyphenols on expression levels of p57, a cyclin dependent kinase and apoptosis inhibitor, in normal human keratinocytes and the oral carcinoma cell lines SCC25 and OSC2. The results showed that the most potent green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCG), induced p57 in normal keratinocytes in a dosage and time dependent manner, while levels of p57 protein in oral carcinoma cells were unaltered. The differential response in p57 induction was consistent with the apoptosis status detected by annexin V assay. The data suggest that the chemopreventive effects of green tea polyphenols may involve p57 mediated cell cycle regulation in normal epithelial cells.

A decade ago, using animal models, green tea was shown to possess an anti-tumor effect (1). Since that time, numerous studies have shown green tea to be chemopreventive in experimental animal and cell culture models. This property has been attributed to the green tea polyphenols (GTPPs) (reviewed in ref. 2). The major polyphenols present in green tea are (-)-Epicatechin (EC), (-)-Epigallocatechin (EGC), (-)-Epicatechin-3-gallate (ECG) and (-)-Epigallocatechin-3- gallate (EGCG) (reviewed in ref. 3). Chemopreventive and therapeutic effects of green tea, and of EGCG's, include: induction of apoptosis in transformed cells but not in normal cells (4, 5, 6); reversible G1 arrest of the cell cycle by inhibition of pRb phosphorylation in pre-cancerous cells (7); modulation of cell cycle regulators in A431

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epidermoid carcinoma cells (8); inhibition of carcinogen/mutagen-DNA binding (9, 10); inhibition of UV induced DNA damage and p53 overexpression (11); and inhibition of UV-induced AP-1 component expression, activity and phosphorylation (12-14). Previous studies have shown that neither GTPPs or EGCG induce apoptosis in the normal keratinocytes used in this study (NHEKs) (15, 16). However, the identification of specific intracellular targets for GTPPs in normal human epithelial cells has been limited to date. Furthermore, the mechanism(s) for the discordant response to GTPPs/EGCG by normal and tumor cells remains unexplained. It is probable that GTPPs/EGCG cause growth arrest in normal cells and pre-cancerous cells by affecting cyclin dependent kinase inhibitors (CKIs) such as p15, p21, p27 and p57, while inducing apoptosis in rapidly replicating tumor cells. Taken together, more information is needed from normal human epithelial cells to understand the chemopreventive effect of GTPPs prior to clinical applications.

In a previous study, we profiled the response of certain cell cycle regulators to GTPPs/EGCG in combination with two tobacco-derived carcinogens, 3,4-benzo (alpha) pyrene (BaP) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in normal human neonatal keratinocytes and the metastatic oral carcinoma cell line OSC2. Western blot analysis showed that GTPPs/EGCG did not significantly induce the expressions of p15, p21and p27. We also found that BaP and NNK altered the expression of certain cell cycle regulators in both normal human epithelial cells and, to a lesser extent, the oral carcinoma cells (17).

In this study, we examined p57 (KIP2) as a potential target for green tea polyphenol regulation. p57 is a potent, p53-independent, tight-binding G1 cyclin/CDK inhibitory protein (18). The human p57 gene is located at chromosome 11p15.5 (19). p57 has been found crucial to embryonic development in mice. Lack of p57 expression resulted in early postnatal death and growth retardation (20). Elevation in p57 expression was associated with intestinal cell differentiation (21). In vitro studies showed induction of p57 leads to a potent growth arrest in G1 with concomitant hypophosphorylation of Rb and diminished E2F-1 (22). To identify whether GTPPs induce p57, we determined p57 protein levels in normal human epithelial cells, an oral carcinoma cell line SCC25 and a metastatic oral carcinoma cell line OSC2 following various treatments using GTPPs.

MATERIALS AND METHODS

Chemicals and compounds: EGCG was obtained from the Lipton Company. A mixture of GTPPs was purchased from LKT Lab. Inc. The carcinogen NNK was purchased from Toronto Research Chemicals, Inc. and BaP was obtained from Sigma. GTPPs and EGCG were dissolved in cell culture media and filter-sterilized immediately prior to use. The NNK and BaP were solubilized with DMSO. Annexin V-EGFP Apoptosis Kit was purchased from CLONTECH Lab. Inc., Palo Alto, CA.

Cell lines and cell culture: The normal human keratinocytes (NHEK CC-2507) were obtained from Clonetics. The SCC25 cell line (Obtained from American Type Culture Collection) was isolated from a squamous cell carcinoma of the tongue of a 70 year-old male (23). The OSC2 cell line (provided by Dr. Tokio Osaki) was isolated from a submandibular lymph node metastasis of a 68-year old female. The primary tumor was located in the gingiva of this patient (24). OSC2 cells have a p53 mutation at exon 8, site 280, resulted in an Arg to Thr conversion (25). SCC25 cells have undetectable p53 levels, while OSC2 cells over-express p53 (17). SCC25 and OSC-2 cells were maintained in 45% Dulbecco's MEM medium (DMEM), 45% Ham's F12 medium and 10% newborn calf serum, 100 Unit/ml penicillin, 100 μg/ml

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streptomycin and 5 µg/ml hydrocortisone. The keratinocytes (two batches were used for repeatability) were cultured and maintained in KGM-2 medium (Clonetics). All cell cultures were maintained in a 37oC incubator with 5% CO_2. For Western blot analysis, the keratinocytes were placed in KGM-D medium (Clonetics) overnight prior to treatment. Rabbit anti-p57 and goat anti-actin antibodies used in this study were purchased from Santa Cruz Biotech Company. Each experiment was repeated at least three times. Three batches of the normal human keratinocytes were tested with consistent results for p57 induction.

Cell treatments: For 24 h treatments, exponentially growing cells with 40% confluency (to minimize differentiation and spontaneous apoptosis) were either maintained in 50 mM EGCG (2.29 mg/100 ml) or 0.2 mg/ml of GTPPs in tissue culture flasks (25 cm2). Control flasks contained cells without any treatment. To test whether BaP or NNK interfere with the induction of p57, the human keratinocytes were treated with 0.12 µM BaP, or 10 µM NNK, either alone or in combination with 50 µM EGCG. To examine the time course of p57 induction, cells were treated with 50 µM EGCG and harvested for Western analysis at 30 min., 2 h, 6 h and 24 h. The human keratinocytes were treated when the cell density reached 85-90% confluency (to mimic the epithelium).

In a parallel series of experiments, treatment with EGCG was performed with 30 µg/ml cycloheximide added to the keratinocyte media 30 min. prior to the addition of EGCG. The dose-response experiments were performed using EGCG concentrations at 30, 50 100, and 200 µM in the culture media for 24 h.

Western blot analysis: Cells from different treatment groups were lysed in RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, and 1% Trasylol) containing proteinase inhibitors (1 mM PMSF, 1 g/ml each of aprotinin, leupeptin, and pepstatin). The concentration of protein in each sample was determined using the BioRad DC Protein Assay and spectrophotometry. 50 µg of protein from each sample and a BioRad molecular weight standard marker were run on a 10% SDS-PAGE, followed by transfer to nitrocellulose membranes. Nonspecific binding to membranes were blocked with 10% nonfat milk. Specific primary polyclonal (rabbit) antibody against p57 and a horse radish peroxidase-conjugated goat anti-rabbit secondary antibody were used in conjunction with the ECL Chemiluminescence Kit and membranes were exposed to radiographic films for detection. Annexin V apoptosis assay: Initially, 10^4 OSC2 cells were seeded in each chamber of an 8-chamber chamberslide and $5X10^4$ human keratinocytes were seeded in each well of a 24-well tissue culture plate. When the cells formed a monolayer in the center, fresh media containing 0.2 mg/ml of GTPPs was added, and 24 h later, the Annexin V assay was performed according to the manufacturer's instructions with minor modifications. Visualization and photography were realized by confocol-fluorescence microscope imaging using a dual filter set for FITC & rhodamine.

RESULTS

Data generated from this study showed a novel observation: p57 was accumulated in normal human epithelial cells, but not in oral carcinoma cells at 40% confluency, following incubation for 24 h with either 50 µM EGCG or 0.2 mg/ml GTPPs (Figure 1A). The keratinocytes consistently showed modestly higher levels of actin (used as a loading control) than the oral carcinoma cells when the same amount of the protein was loaded. When BaP and NNK were present, EGCG-stimulated p57 protein accumulation still occurred in the epithelial cells, although at slightly lower levels compared to EGCG alone (Figure 1B). BaP and NNK

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alone did not alter p57 expression, as shown in lanes 2 and 3. When the normal epithelial cell density was 90%, p57 expression induced by 50 mM EGCG reached its peak at 6 h, and declined to basal levels by 24 h (Figure 2A). This contrasts with the results obtained at 40% confluency (Figure 1A) where p57 levels remained high. However, when the EGCG concentration was increased to 100 μM or 200 μM, p57 levels in cells at 90% confluency remained high throughout the 24 h period examined (Figure 2B). Thus, the induction of p57 by EGCG is dependent on the dose, time and confluency of the cells. Treatment with cycloheximide resulted in a gradual decline in p57 protein levels, while actin levels remained relatively constant, indicating that in normal human keratinocytes, the p57 protein accumulation induced by 50 μM EGCG was the result of new synthesis instead of decreased degradation (Figure 2A, EGCG + Chx). The metastatic oral carcinoma OSC2 cells failed to elevate p57 expression in response to EGCG at any time point or concentration (Figure 3). The annexin V apoptosis detection assay demonstrated that 0.2 mg/ml GTPPs induced differential response in apoptotic status from the normal epithelial cells and the oral carcinoma cells. Only OSC2 cells treated with 0.2 mg/ml GTPPs exhibited apoptosis detected by green colored annexin V-FITC associated with phosphatidylserine on the outer cell membrane and broken cell membrane. The human keratinocytes, both control and GTPPs treated, did not show phosphatidylserine translocation or broken cell membranes, nor did OSC2 control. As indicated by annexin V-FITC, which binds to apoptotic cells that expose phosphatidylserine molecules on the outer layer of the cell membrane, OSC2 cells treated with GTPPs for 24 h showed massive apoptosis, compared with untreated cells. In contrast, the keratinocytes did not exhibit any phosphatidylserine translocation in the control nor in GTPPs treated samples.

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DISCUSSION

It is a significant observation that a group of plant derived compounds can induce a cell cycle regulator in normal human cells in a time and dose dependent manner. Therefore we report here, for the first time, that p57 (KIP2), a CDK and apoptosis inhibitor, is an intracellular/nuclear target for green tea polyphenols in normal human epithelial cells (keratinocytes), but not in the tumor cells tested. Furthermore, the tobacco carcinogens BaP and NNK do not markedly inhibit this p57 induction (Figure 1B). But the long-term effect of BaP and NNK to the p57 induction is not known. p57 induction in the normal epithelial cells showed remarkable correlation with apoptosis resistance. This correlation of p57 induction (Figure 1A) and resistance to GTPPs-induced apoptosis only in normal human keratinocytes suggests that p57 may be involved in mechanisms that enhance cell survivability during GTPPs treatment. The N-terminus of p57 protein is able to bind CDK-cyclin complex and inhibit its kinase activity; the C-terminus of p57 protein contains a PCNA (proliferating cell nuclear antigen)-binding domain that suppress cell proliferation (26). It is possible that elevated p57 protein expression may induce reversible growth arrest in the normal keratinocytes and prevent E2F mediated apoptosis through hypophosphorylation of Rb protein (22). In contrast, oral carcinoma cell lines derived from a primary site and from a metastasis failed to elevate p57 expression, and would be unable to survive the apoptotic effect of GTPPs/EGCG. This differential response in p57 induction may help us to explain why green tea polyphenol-mediated apoptosis has been found only in tumor cells (4, 5, 6, 27, 28). In addition, EGCG concentration and cell density are two determinant factors for the duration of each p57-induction cycle. Future investigations should include models that resemble human epithelial/endothelial tissues to extend our understanding of the chemopreventive effects of GTPPs through induction of p57

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expression. These effects could simultaneously protect the normal cells and induce apoptosis in tumor cells by other mechanism(s). We have reported that EGCG inhibited the growth and invasion of metastatic oral carcinoma OSC2 cells (17). The SCC25 cells were reportedly inhibited by GTPPs (29). Activation of caspase 3 by EGCG in tumor cells was first reported in human chondrosarcoma cells with simultaneous and dosage dependent growth inhibition and apoptosis (6). Studies on human cervical squamous carcinoma HeLa cells showed 25 μM EGCG activated caspase 3 activity in 16 h. This activation was accompanied by time- and dose-dependent activation of mitogen-activated kinases ERK, JNK and p38 (30). A recent report showed green tea extract induced cytochrome c release and caspase-3-like protease activation in Ehrlich ascites tumor cells (31). Thus, accumulating evidence suggests that GTPPs selectively induce caspase 3 mediated apoptosis in tumor cells. Since loss of p57 expression/induction often occurred in tumor cells, such cells would undergo GTPPs/EGCG-induced apoptosis (32, 33). Conversely, non-cancerous cells such as T-lymphocytes protect themselves from apoptosis by elevation of p57 (34). Therefore p57 elevation could be responsible for normal keratinocyte cell survival when exposed to GTPPs. This hypothesis is currently being tested in our laboratories. We conclude that a group of plant derived compounds, green tea polyphenols, are able to induce p57 protein expression in human keratinocytes but not in two oral cancer cell lines tested. This induction is dosage- and time-dependent. It is apparent that exposure to GTPPs/EGCG reversibly increases the level of intracellular p57 expression in the normal human epithelial cells tested, which may serve as a dual-effect chemopreventive mechanism. When cells lose the p57 response as in tumor cells, these cells would be defenseless and selectively induced to apoptosis by GTPPs/EGCG.

Therefore, frequent consumption of green tea or green tea polyphenols may contribute to chemoprevention against oral cancer.

LEGENDS OF FIGURES

Figure 1. A. Differential p57-induction demonstrated by Western analysis of whole cell lysates from human keratinocytes, SCC25 and OSC2 human oral carcinoma cells at 40% confluency. Only the keratinocytes responded to EGCG and GTPPs by elevation of p57. Cells were treated for 24 hr as follows: C. Control; E. 50 μM EGCG; G. 0.2 mg/ml GTPPs. p57 levels in SCC25 and OSC2 cells remained unchanged. B. p57 induction in Western analysis of whole cell lysates from human keratinocytes treated under different conditions: 1. Control; 2. BaP; 3. NNK; 4. EGCG; 5. EGCG + BaP; 6. EGCG + NNK. The relative densities of the p57 bands were compared on Western blots using the UTHSCSA Image Tool imaging software. The blot image was converted from color to grayscale and the band density measured on a scale of 1-255 densitometric units/ mm2. Lane 4 (EGCG treated) represents a 12-fold increase comparing to lane 1 (Control).

Figure 2. A. Reversible p57 induction in Western analysis of whole cell lysates from human keratinocytes in a time course experiment with 85-90% cell density. C (lane 1): 24 h untreated cells as control; EGCG (lanes 2-5): cells treated for the indicated length of time with 50 μM EGCG; EGCG + Chx (lanes 6-9): cells treated for the indicated length of time with 50 μM EGCG and 30 μg/ml cycloheximide. B. Increasing p57 expression in Western analysis of whole cell lysates from human keratinocytes on dose response experiment with 85-90% cell density. Cells were treated with indicated concentrations for 24 h prior to harvesting.

Figure 3. Lack of p57-induction demonstrated by Western analysis of whole cell lysates from OSC2 cells. Darker background with p57 bands was due to extended exposure time following ECL reaction (10 min). Lane 1-4 are samples treated with indicated concentration of EGCG for 24 h. Lane 5 (C) contains control sample without any treatment, lanes 6-9 contain samples treated with 50 μM EGCG for the indicated length of time. The nitrocellulose membrane was hybridized with anti-p57 antibody followed by hybridization with anti-human actin antibody.

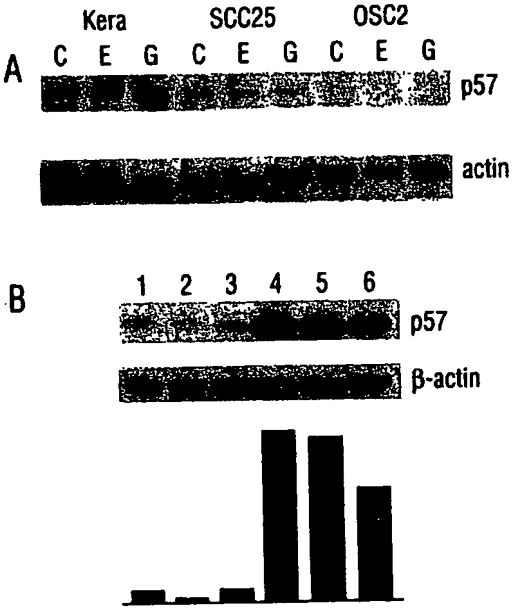

Figure 1. *A. Differential p57-induction demonstrated by Western blot analysis of whole cell lysates from human keratinocytes, SCC25 and OSC2 human oral carcinoma cells at 40% confluency. Only the keratinocytes responded to EGCG and GTPPs by elevation of p57. The cells were treated for 24 hours as follows: C. Control, E. 50 μM EGCG; G 0 2 mg/ml GTPPs. p57 levels in SCC25 and OSC2 cells remained unchanged. B. p57 induction in Western blot analysis of whole cell lysates from human keratinocytes treated under different conditions: 1. Control; 2 BaP, 3. NNK; 4 EGCG; 5 EGCG + BaP; 6 EGCG + NNK The relative densities of the p57 bands were compared on Western blots using the UTHSCSA Image Tool imaging software The blot image was converted from color to gray scale and the band density measured on a scale of 1-255 densitometric units/ mm^2. Lane 4 (EGCG-treated) represents a 12-fold increase compared to lane 1 (Control)*

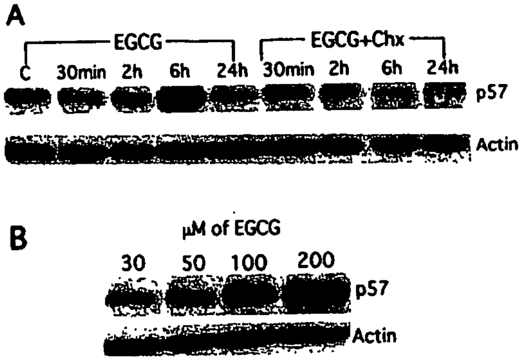

Figure 2. *A Reversible p57-induction in Western blot analysis of whole cell lysates from human keratinocytes in a time course experiment with 85-90% cell density. C (lane 1): 24-hour untreated cells as control, EGCG (lanes 2-5). cells treated for the indicated length of time with 50 μM EGCG; EGCG + Chx (lanes 6-9) cells treated for the indicated length of time with 50 μM EGCG and 30 μg/ml cycloheximide. B Increasing p57 expression in Western blot analysis of whole cell lysates from human keratinocytes on dose response experiment with 85-90% cell density The cells were treated with indicated concentrations for 24 hours prior to harvesting.*

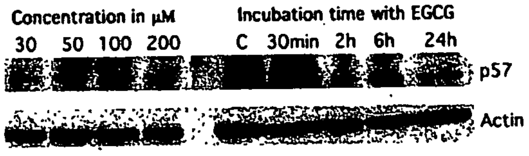

Figure 3. *Lack of p57-induction demonstrated by Western blot analysis of whole cell lysates from OSC2 cells The darker background with p57 bands was due to extended exposure time following ECL reaction (10 minutes). Lanes 1-4 are samples treated with indicated concentration of EGCG for 24 hours. Lane 5 (C) contains control sample without any treatment while, lanes 6-9 contain samples treated with 50 μM EGCG for the indicated length of time. The nitrocellulose membrane was hybridized with anti-p57 antibody followed by hybridization with anti-human actin antibody.*

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Example 2

Chemoprevention of Oral Cancer by Green Tea

Cancer is the second leading cause of death in the United States following only to cardiovascular diseases. More than 30,000 patients will be diagnosed with oral cancer with an estimated 7800 deaths (1). The rates of oral cancer have been decreasing since early 1980's; nevertheless, a rather static five year mortality rate of 53% to 56% has been reported during the past few years (1-4). Furthermore, the disfigurement from surgical treatment of these cancers often results in prolonged trauma in patients even after the disease has been controlled. The highest rates of oral cancer are in developing countries such as South Asia and Southeast Asia where oral cancer is the first or second most common malignancy (5, 6). The incidence of oral cancer in Europe and United States is ~ 2-6%, whereas in some parts of India, it accounts for 30-40% and is regarded as a 'new epidemic' (7-9).

The risk factors for oral squamous cell carcinoma include primarily smoking (cigarettes, cigars, pipe), smokeless tobacco (chewing tobacco and snuff), and alcohol, which has a synergistic effect with smoking (2, 10-12). In addition, the data regarding the role of viruses (13) oncogenes (14), and sun light have also been forthcoming (15). The differential oral cancer incidence among countries, and even among populations in the same country may reflect variations in the etiologic factors, tumor promoters and their interaction with dietary constituents, habits, genetics, environment and hygiene.

The use of smokeless tobacco has been associated with the development of oral precancerous and cancerous lesions. The types of chewing tobacco and snuff commonly used in North America and European countries are considered carcinogenic and risk factors for

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development of oral cancer (16, 17). A classical study estimated that 1/3 of the oral neoplasms were attributable to the use of snuff (18). The available smokeless tobacco products in the United States and European countries generally do not contain significant additional materials commonly designated as 'additives', compared to specific global areas with a high rate of oral leukoplakia and cancer. For example, smokeless tobacco in combination with additives such as Areca nut, Betel nut, Betel leaf, Catechu and Cardamom is strongly associated with development of oral leukoplakia and cancer in India (16, 19,-21). The progression of leukoplakia, potentially considered a premalignant lesion, to carcinoma is unpredictable. Homogenous leukoplakia exhibits a malignant transformation rate of approximately 7%, while transformation rates for erythroleukoplakia and epithelial dysplakia are estimated at 23% and 36%, respectively (2). Oral cancer is known to exhibit 'Field Cancerization' (22), and the patients subsequently develop a second primary tumor at an annual rate of 4%-7% (2).

It is evident that smoking is one of the etiological factors in the development of oral cancer in the United States (10). However, there is still no explanation as to why China, which has a population of heavy smokers and poor oral hygiene, has a far lowest incidence of oral cavity, lip, and pharyngeal cancers in males (4.66/100000) compared to North America (11.69/1000000) and South Central Asia (20.5/100000) (7, 8). The Chinese population is unique in its high consumption of green tea. Drinking tea has been a tradition for many centuries; in fact more than 2/3 of the world population consumes this most popular beverage, but majority of tea consumed in the world is black tea (78%), not green tea (20%) (23).

In epidemiological studies, green tea consumption appears to lower the risk of gastro-intestinal cancer (24), esophageal cancer (25) and decrease lymph node metastasis of breast cancer (26). Some studies have associated with an increased risk of cancer with tea intake, but inconsistencies have been attributed to the consumption of very hot or salty tea in specific geographic locations, as well as other confounding factors, which may include use of tobacco and alcohol in these geographical locations. In animal studies, green tea and its compounds were found to possess preventive or therapeutic effects against lung cancer (27, 28), prostate cancer (29, 30), and urinary bladder cancer (31).

This brings in focus the role of putative chemopreventive agents such as green tea, which can act in local oral environments as well by systemic absorption. Cancer chemoprevention has been defined as the use of agents to slow the progression of, inhibit, delay or reverse carcinogenesis (32). The efficacy and application of chemopreventive agents depends upon the identification of target molecules. For example, signal transduction modulators, oncogene inhibitors and retinoids have shown significant chemopreventive activity. The potential roles of cell cycle regulators are germane to our investigations (33, 34). Green tea appear to be chemopreventive both before the onset of malignancy, and following cancer treatment (35). Recent human studies demonstrated that green tea consumption reduced oxidative DNA damage and free radical formation in smokers and non-smokers (36, 37).

Green tea was found to possess anti-tumor effects in animal models a decade ago (38). Since then, green tea has been proven chemopreventive against carcinogenesis/tumorigenesis in mammals (29, 26, 31) and cell culture models (39). This property has been attributed to its antioxidant components, green tea polyphenols (GTP) (40, 41 and 42 for review). The four major polyphenols present in green tea are (-)-Epicatechin (EC), (-)-Epigallocatechin (EGC), (-)-Epcatechin-3-gallate (ECG) and (-)-Epigallocatechin-3-gallate (EGCG) (43 for review), among which, EGCG has been tested to be able to access organs throughout the body (44).

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Green tea inhibits growth of many types of human cancer cells (39, 45-47). On the molecular level, GTP/EGCG were found to inhibit: UV-induced DNA damage and p53 tumor-suppressor gene overexpression (48); carcinogen/mutagen-DNA binding (49, 50); as well as inhibition of oncogene expression and mitogen-activated protein kinase (MAPK) activation (51). Other effects ascribed to GTP or EGCG include inhibition of tumor necrosis factor alpha (TNF-alpha) gene expression and TNF-alpha release from cells (26), inhibition of tumor promoter-induced AP-1 activation and tyrosine phosphorylation of PDGF beta receptor (52). EGCG was also found to induce caspase-3 activated apoptosis (programmed cell death) in tumor cells (53, 54).

In addition, evidence showed that GTP/EGCG at concentrations that induced apoptosis in tumor cells, failed to induce apoptosis in normal human keratinocytes (53, 55). It is suggested that GTP differentially directs the fates of normal and tumor cells. Although there is some evidence for protective effects of green tea against oral lesions, studies on the effects of green tea on oral lesions are limited (56), and mechanism(s) for the distinct survival/death responses to GTP/EGCG by normal and tumor cells remain unexplained.

Therefore, the current investigation was designed to investigate the effect of GTP/EGCG on normal keratinocytes and oral squamous cell carcinoma (SCC) cells in order to elucidate molecular parameters that might be protective for normal cells and cause cell death in neoplastic cells. These observations would provide a scientific basis for green tea as a bona fide chemopreventive agent for oral malignancy and justify its institution as a safe public health strategy.

Cancer is increasingly viewed as a cell cycle disease (33 for review). In eukaryotes, the cell cycle is controlled by a number of cell cycle regulators (57) such as cyclin dependent kinases (CDKs) and CDK inhibitors (CKIs) (58-61). CKIs regulate the cell cycle by imposing

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growth arrest. When growth arrest occurs in normal human keratinocytes, these cells became resistant to apoptosis signals such as UV light (62). Previously published reports did not show significant induction of cell cycle regulator proteins by GTP/EGCG, which may be ascribed to limited data available in normal human epithelial systems. We have profiled certain CKIs in response to GTP/EGCG in both normal epithelial cells and tumor cells (63) and made a novel observation: one of the CKIs, p57 (KIP2), can be specifically induced by GTP/EGCG only in normal human epithelial cells (64). The significance of this finding is that a group of plant-derived compounds are able to specifically induce a human gene product in a dose and time-dependent manner for either cell survival or apoptosis.

Based on our observations, we hypothesize that green tea polyphenols should induce p57 in normal epithelial cells, serving an anti-apoptosis function; in tumor cells, failure to elevate p57 levels in the presence of the polyphenols may result in induction of caspase 3 (the key limiting enzyme for apoptosis) dependent apoptosis.

MATERIALS AND METHODS

Chemicals and compounds: EGCG was kindly provided by Dr. Mohammad Athar (Columbia University). A mixture of GTP was purchased from LKT Lab. Inc. Annexin V-FITC Apoptosis Kit was purchased from CLONTECH Lab. Inc., Palo Alto, CA. Crude green tea extract was prepared by incubation of 4 ml cell culture media with a green tea bag (P.R.I., NJ) for 10 min followed by collection of the extract.

Cell lines and cell culture: The normal human keratinocytes (NHEK CC-2507) were obtained from Clonetics. The SCC25 cell line (Obtained from American Type Culture Collection) was originally isolated from a squamous cell carcinoma of the tongue of a 70 year-old male (65). The OSC2 cell line (provided by Dr. Tokio Osaki) was isolated from a submandibular lymph node metastasis of a 68-year old female. The primary tumor was located in the gingiva of this patient (66). OSC2 cells have a p53 mutation at exon 8, site 280, resulted in an Arg to Thr substitution (67). SCC25 cells have undetectable p53 levels, while OSC2 cells over-express p53 (63). The DOK cell line is a dysplastic immortal oral keratinocytes cell line (68). SCC25, DOK and OSC2 cells were maintained in 45% Dulbecco's MEM medium (DMEM), 45% Ham's F12 medium and 10% newborn calf serum, 100 Units/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml hydrocortisone. The keratinocytes were cultured and maintained in KGM-2 medium (Clonetics). All cell cultures were maintained in a 37oC incubator with 5% CO_2

Annexin V apoptosis assay: Initially, 10^4 of tumor cells were seeded in each chamber of an 8-chamber chamberslide and $5x10^4$ human keratinocytes were seeded in each well of a 24-well tissue culture plate, and the monolayers were subjected to 24 h-0.2 mg/ml of GTP treatment, followed by the Annexin V assay according to the manufacturer's instructions with minor modifications.

Cell growth assay: Cells $(2x10^5)$ were seeded in each T25 culture flask with 5 ml DMEM/F12 medium for 48 hrs. The treatments were started with 50 μM EGCG for 24 h, 48 h and 96 h. At each time point, the cell numbers were counted using a hemacytometer with the presence of Trypan blue.

Cell invasion/migration assay: The invasion/migration assays were conducted using a Transwell apparatus (Costar) with 6.5 mm diameter wells and membranes of 8 μm pore size. The invasiveness at each time point was tested in DMEM/F12 medium immediately following the cell growth assay, by seeding 10^5 cells in each transwell. Cells migrated across the transwell membrane were counted as per microscopic field.

RESULTS AND DISCUSSION

It is evident that green tea is a powerful inducer of apoptosis in tumor cells. One hour incubation of a small percentage of green tea crude extract (80 μl/5 ml) was able to induce morphological change in OSC2 cells (Fig. 1B) comparing to untreated control (Fig. 1A). Two 1-h incubation of the crude extract separately within a 24 h period further increased the number of dead cells (Fig. 1C). When green tea crude extract at 125 μl/5 ml was continuously incubated with OSC2 cells for 6 h (Fig. 1E) and 24 h (Fig. 1F), the majority of the cells underwent cell death comparing to the control (Fig. 1D), and cells from Fig. 1F were not able to recover when they were placed back to normal media (data not shown). This result suggested that exposure to green tea could lead to elimination of oral cancer (squamous cell carcinoma) cells. Based on this observation, we applied 0.2 mg/ml GTP on a oral cancer progression model system that consists of normal human epithelial cells (pooled newborn epidermal keratinocytes), a pre-cancerous dysplastic oral keratinocyte cell line DOK, a primary oral carcinoma line SCC25, and a metastatic oral carcinoma line OSC2. To examine the status of apoptosis, 0.2 mg/ml GTP was incubated with exponentially growing cells for 24 h followed by Annexin V apoptosis assay. As indicated by the presence of annexin V-FITC (green), OSC2 and SCC25 cells treated with GTP for 24 h showed massive apoptosis (Fig. 2F and H), compared with untreated cells (Fig. 2E and G). In contrast, normal epithelial cells did not exhibit any apoptotic cells in the control (Fig. 2A) nor in GTP treated samples (Fig. 2B), and there was no massive apoptosis in DOK cells (Fig. 2D). This result indicated that GTP differentially induced apoptosis in oral cancer cells and the apoptosis pathway was not p53 dependent, since OSC2 cells have a mutated and overly expressed p53, and SCC25 cells do not express p53. To further investigate this property of GTP, the most potent component, EGCG, was used at a lower concentration (50 µM, which is 1/7 w/w of that of 0.2 mg/ml GTP) to determine its impact on OSC2 cells. EGCG was effective in inhibiting cell growth within 24 hrs. By day four, the number of EGCG-treated OSC2 cells were only 50% compared to the controls as shown in Figure 3A. Inhibition of cell invasiveness/migration was rapid. Figure 3B shows that after 24 hr of treatment, cells invading the membrane were reduced to about 30% of control. Following 96 hr of treatment, the percentage was further reduced to 20%. These data suggest that EGCG is able to both reduce the mobility of metastatic oral cancer cells and inhibit their growth. Based on the observations that GTP/EGCG induced a differential response between normal and oral cancer cells, we next searched for potential intracellular targets of GTP/EGCG. We observed that a cyclin dependent kinase inhibitor p57 was significantly altered only in the normal cells in response to GTP/EGCG. To determine whether p57 is an intracellular target for GTP/EGCG, Western analysis was performed using the normal human keratinocytes and two oral carcinoma cells lines SCC25 and OSC2 in a separate study (69). The result showed that EGCG specifically induced p57 in normal keratinocytes, while levels in oral carcinoma cells were unaltered. Treatment of normal human keratinocytes at 40% confluency with 50 µM of EGCG induced up to a 12-fold increase of p57 expression, and the induction of p57 expression is time- and dose-dependent. In contrast, OSC2 cells (and several squamous cell carcinoma lines examined) failed to elevate p57 expression in response to EGCG at any time point or concentration. Therefore, p57 could serve as a target for GTP/EGCG in the normal epithelial cells to initiate a survival mechanism while oral cancer cells lacking the p57 response would undergo the apoptosis pathway.

Taken together, when normal epithelial cells (with the p57 response) are exposed to green tea/GTP/EGCG, induction of p57 (accompanied with other possible events) would enable the cells to survive, possibly through growth arrest or differentiation. On the other hand, oral carcinoma cells (without the p57 response) would undergo a specific apoptosis pathway. Our on-going study indicates that lack of a p57 response to EGCG leads to mitochondria-mediated, caspase 3 dependent apoptosis.

A higher consumption of fruits and vegetables was associated with reduced risk and a protective effect of a number of systemic cancers including those of the oral cavity and pharynx (70-74). Active ingredients in fruits and vegetables that may affect the development of oral precancer and cancer include antioxidants (Beta-carotene, Vitamin A, Vitamin C, Vitamin E, Folic Acid and Lycopene, a tomato constituent). Other active agents of interest include phytochemicals such as flavonoids (in soy beans), Acanthocyanins (in grapes, red cabbage), Ellagic Acid (in raspberries, strawberries, cranberries), Glucosinolates (in cruciferous vegetables), polyphenols (in Green Tea) and others (75). In addition, multiple antioxidant vitamins apparently reduced the size of oral leukoplakic lesions (reviewed in 76).

The results of recent clinical studies illustrate a change in emphasis in cancer chemoprevention toward a mechanism-derived hypothesis. At this juncture, our data suggest a strong potential for green tea and/or its constituents (EGCG) to combat oral malignancy, including precancer and oral cancer. We propose that green tea polyphenols are able to activate two pathways: 1) survival through p57 induction, and 2) caspase 3-dependent apoptosis without p57 induction. We hypothesize that p57 induction by green tea polyphenols in normal epithelial cells may serve as an anti-apoptotic function. Lack of the p57 stimulatory response to the presence of the polyphenols may result in induction of caspase 3-dependent apoptosis (Fig.4). In conclusion, the nature of the chemopreventive effects of green tea may rely on its ability to signal a given cell and trigger a specific gene/cellular response, which directs the cell to undergo either survival or apoptosis pathway.

Based on our laboratory observations and those reported in literature, the clinical application of GTP/ECGC as an anti-cancer agent warrant further investigations. The molecular studies in conjunction with the available epidemiological data should provide the foundation for future clinical trials. Nevertheless, we suggest that the use of green tea as a beverage or in oral health/skin care products for the US population at large is likely to be beneficial.

FIGURE LEGENDS

Figure 1. Morphological change was observed when OSC2 cells were exposed to green tea crude extract (25 μl/ml) for 1 h during a 12 h period (B) in comparison with untreated cells (A). When these cells were exposed to green tea crude extract for a second 1 h incubation within a 24 h period, apoptotic cells were apparent with reduced size, loss of contact and uncharacterized nuclei (C). When green tea crude extract (16 μl/ml) was incubated un-interruptedly with oral carcinoma cell line OSC2 for 6 h, many cells were disfigured and detached (E), by 24 h, massive cells death was observed and increased debris from cell lysis (F) in comparison to the untreated cells (D). Magnifications: 400x for A, B and C; 100x for D, E and F.

Figure 2. A. EGCG inhibits growth of OSC2 cells. OSC2 cells were incubated with 50 μM EGCG for 24, 48 and 96 h, and cell number were counted in comparison with the cell number of untreated control. B: Inhibition of invasiveness of OSC2 cells by EGCG treatment. After 24, 48, 96 hrs of treatments with EGCG, cells (10^5) were loaded onto each transwell of a

24-well transwell plate. Both tests were conducted three times with similar results. The controls are presented as 100% in cell number. C. Control cells invaded. D. Cells invaded after 96 h EGCG treatment.

Figure 3. Annexin V apoptosis assay showing normal keratinocytes (A, B), pre-cancerous dysplastic oral keratinocytes DOK (C, D), metastatic oral carcinoma cells OSC2 (E, F), and primary oral carcinoma cells SCC25 (G, H) before (left column) and after 24 h incubation with 0.2 mg/ml GTP (right column). Apoptosis coupled with trans-localization of phosphatidylserine was visualized by annexin V linked with FITC fluorescent dye (green). OSC2 cells and SCC25 cells showed massive apoptosis. SCC25 photos were enlarged to demonstrate detailed images of cell membrane and the nuclei.

Figure 4. Schematic model for the dual-effects of green tea polyphenols, which differentially target between normal and tumor cells. Either survival pathway or apoptotic pathway could be activated, depending on whether p57 protein production is induced. Induction of p57 appears to inhibit the apoptotic pathway. C3 represents caspases 3.

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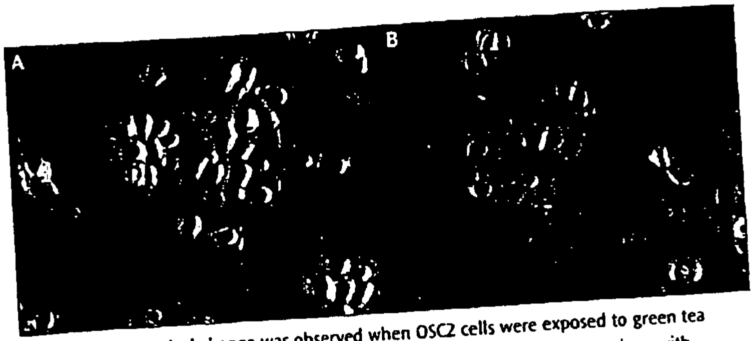

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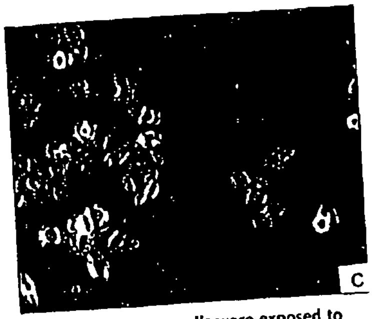

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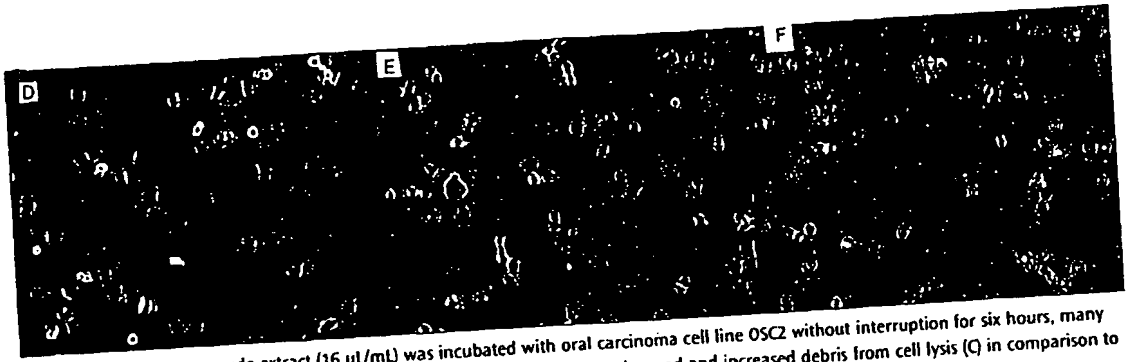
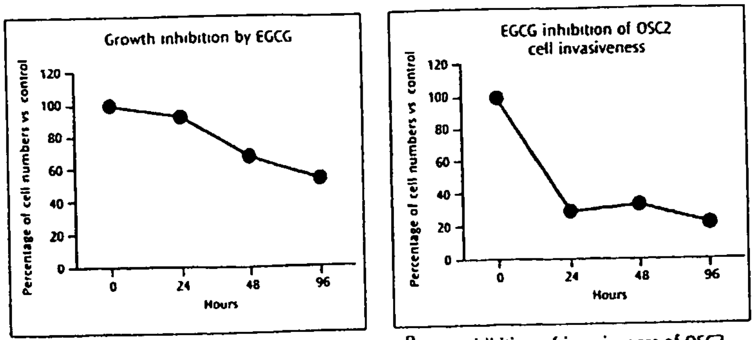


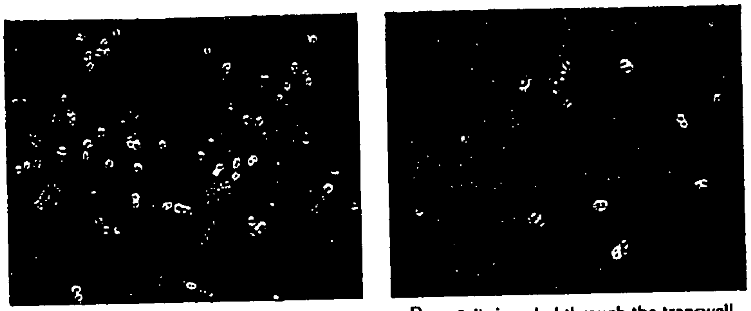
Fig. 1 When green tea crude extract (16 µL/mL) was incubated with oral carcinoma cell line OSC2 without interruption for six hours, many cells were disfigured and detached (B); by 24 hours, massive cell death was observed and increased debris from cell lysis (C) in comparison to the untreated cells (A). (Magnification 100X)

Figure 2



A Growth inhibition of OSC2 cells by EGCG.

B Inhibition of invasiveness of OSC2 cells by EGCG treatment.



C Control cells invaded into the lower compartment of the transwell.

D Cells invaded through the transwell after 96 hours of EGCG treatment.

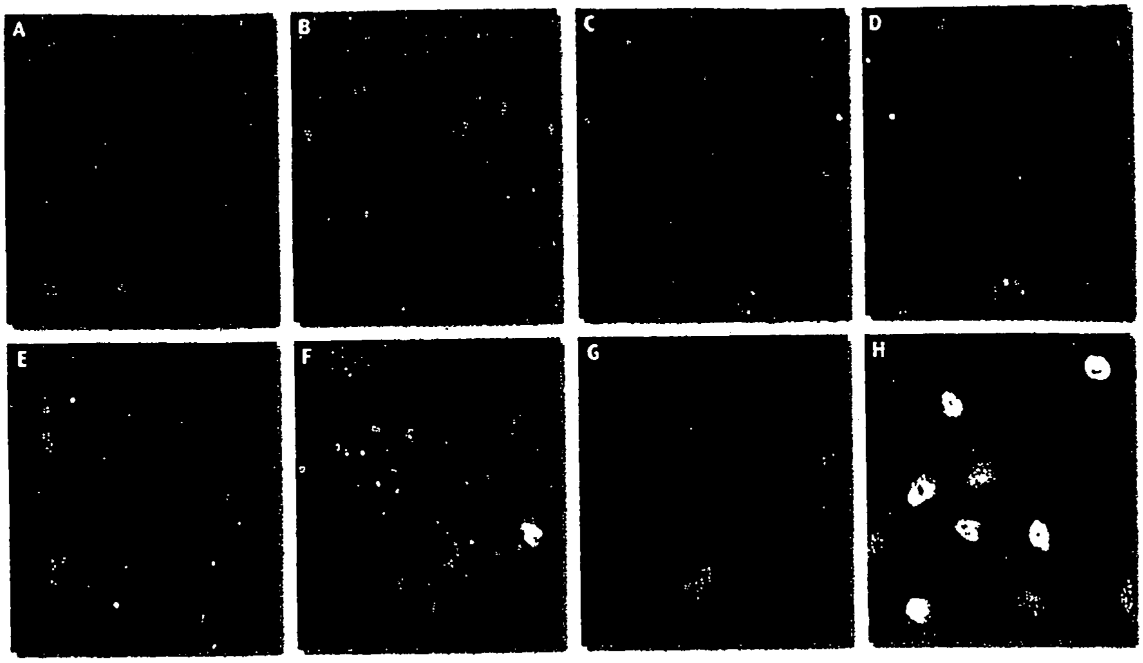


Fig. 3 Annexin-V apoptosis assay showing normal keratinocytes (A, B), precancerous DOK (C, D), metastatic oral carcinoma cells OSC2 (E, F), and primary oral carcinoma cells SCC25 (G, H) before (A, C, E, G) and after (B, D, F, H) 24 hours incubation with 0.2 mg/mL GTP. Apoptosis coupled with trans-localization of phosphatidylserine was visualized by annexin-V linked with FITC fluorescent dye (green) OSC2 cells and SCC25 cells showed massive apoptosis. SCC25 photos were enlarged to demonstrate detailed images of cell membrane and the nuclei.

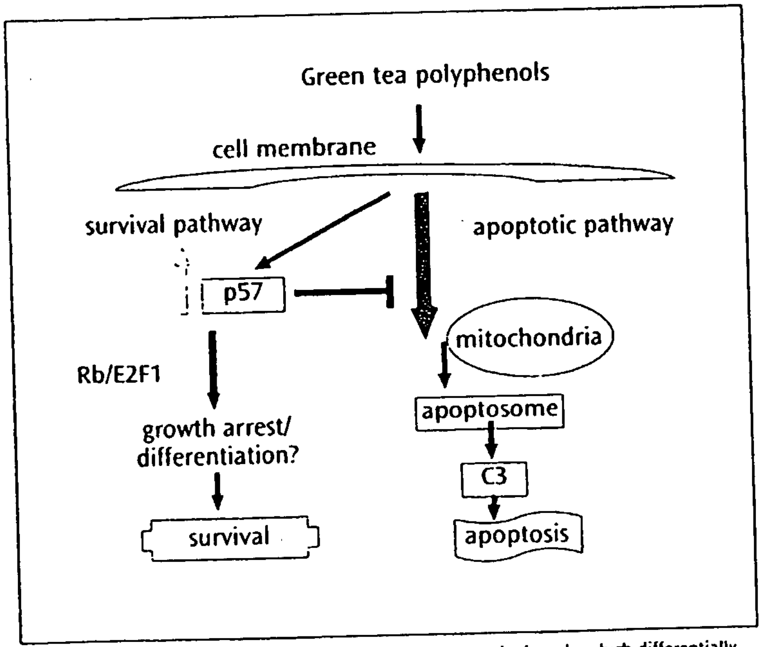


Fig. 4 Schematic model for the dual effects of green tea polyphenols, which differentially target between normal and tumor cells Either survival pathway or apoptotic pathway could be activated, depending on whether p57 protein production is induced. Induction of p57 appears to inhibit the apoptotic pathway C3 represents caspase-3

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Example 3

Induction of p57 Is Required for Cell Survival When Exposed to Green Tea Polyphenols

Green tea polyphenols (catechins) are known to induce cell death in many types of tumor cells, but how normal epithelial cells survive in the presence of polyphenols is unknown. We recently reported that green tea polyphenols potently induced a cyclin dependent kinase inhibitor, p57/KIP2, only in normal human epithelial cells. In this study, we investigated the correlation between p57 expression and survival/apoptosis by Western blot analysis, caspase 3 assays and morphological analysis. It was demonstrated that in the cells that lack p57 induction, green tea polyphenols induced Apaf-1 expression along with caspase 3 activation, leading to apoptosis. In contrast, cells with polyphenol-inducible p57 maintained constant levels of Apaf-1 and proliferating cell nuclear antigen (PCNA), with basal caspase 3 activity.

Retroviral-transfected, p57-expressing oral carcinoma cells showed significant resistance to green tea polyphenol-induced apoptosis. Our results suggest that p57/KIP2 is a determinant pro-survival factor for cell protection from green tea polyphenol-induced apoptosis.

As a potential alternative medicine, green tea polyphenols (GTPPs) have been found to be chemopreventive against certain cancers. However, by what mechanism(s) the normal cells survive this polyphenol exposure is not known. It was found that p57/KIP2 induction is associated with cell survival of epidermal keratinocytes exposed to green tea polyphenols at concentrations that otherwise would cause apoptosis in tumor cells (1). The p57 gene product is a potent, p53 independent, tight-binding G1 cyclin/CDK inhibitory protein (2). The C-terminus of p57 protein possesses a binding domain for PCNA (3). Embryonic development in mice requires p57 expression; absence of it resulted in early postnatal death and growth retardation (4, 5). On the other hand, in human intestinal cell models, elevation of p57 expression was associated with intestinal cell differentiation (6). T-lymphocytes protect themselves from apoptosis by maintaining high levels of p57 (7). Recent pathological studies demonstrated that tumor specimens express lower levels of p57 protein compared to paired normal tissues, and low levels of p57 often correlate with poor prognosis (8-11). In vitro studies using human astrocytoma cells showed that induction of p57 led to growth arrest in G1, with concomitant hypophosphorylation of Rb and diminished E2F-1 (12). Therefore, it appears that p57 plays an important role in inhibition of apoptosis, since at least two apoptotic pathways can be activated by E2F independent of p53, through activation of p73 (13-15) or apoptotic protease activating factor-1 (Apaf-1) (16). Both pathways require cytochrome c release from the mitochondria and apoptosome formation, which consists of cytochrome c, procaspase 9 and oligomerized Apaf-1 (17). Apaf-1 was first identified in 1997 (18) and proved to be a limiting key factor for mitochondrion-mediated apoptosis (19). Binding with cytochrome c activates Apaf-1; it hydrolyses ATP or dATP to oligomerize into a large complex. This complex then binds and activates procaspase 9 and subsequently initiates the caspase pathway towards apoptosis (17). Cells without Apaf-1, such as certain malignant melanomas, are resistant to chemotherapy (20). It was suggested that p57 induction by green tea polyphenols, especially epigallocatechin-3-gallate (EGCG), lead to a cell survival pathway (21). In order to determine whether cells lacking p57 response would fail to survive the EGCG challenge, we compared two normal human cell types, a mammary epithelial cell population from one individual without the p57 response to EGCG treatment, and pooled epidermal keratinocytes that respond to EGCG by p57 induction. Furthermore, retroviral-transfected, p57-expressing metastatic oral squamous cell carcinoma OSC2 subclones also were examined to evaluate the impact of p57 expression in response to green tea polyphenol-induced apoptosis.

MATERIALS AND METHODS

Chemicals and antibodies: EGCG was purchased from Sigma (St. Louis, MO). A mixture of four major GTPPs was purchased from LKT Lab. Inc (Minneapolis, MN). GTPPs and EGCG were dissolved in cell culture medium and filter-sterilized immediately prior to use. Rabbit anti-human p57, Apaf-1, PCNA and goat anti-human Actin antibodies used in this study were purchased from Santa Cruz Biotech Company (Santa Cruz, CA).

Cell lines and cell culture: The normal human keratinocytes (NHEK CC-2507) were purchased from Cambrex (East Rutherford, NJ) and maintained in KGM-2 medium (Cambrex). The OSC2 cell line was previously described (1). The OSC2 subclones were established by retroviral transfection of the parental OSC2 cell line (details of establishing p57-expressing OSC2 subclones and their response to GTPPs will be reported in a separate communication). These clones were maintained in 45% Dulbecco's Modified Eagle's Medium (DMEM), 45%Ham's F12 medium and 10% fetal calf serum, 100 Unit/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml hydrocortisone. The normal human mammary epithelial cells (HMEC) were maintained in MEGM medium (Cambrex). All cell cultures were maintained in a 37o C incubator with 5% CO_2 light photographs were taken with a SPOT RT digital camera system (Diagnostic Instruments) linked to a Nikon Phase Contrast-2 microscope at an original magnification of 400X. Fluorescent photomicrographs were taken with a SPOT color digital camera system using the ZEISS Axiovert 10. Fluorescence was generated by a ZEISS AttoArc 2 source with an original magnification of 400X. Experiments were repeated three times.

Western blot analysis: The keratinocytes and the mammary epithelial cells were placed in KGM-2 and MEGM, respectively, overnight prior to treatment. Cells were lysed, after 24 hr treatment, in RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, and 1% Trasylol) containing proteinase inhibitors (1 mM PMSF, 1mg/ml each of aprotinin, leupeptin, and pepstatin). The concentration of protein in each sample was determined using the BioRad DC Protein Assay and spectrophotometry. 50 µg of protein from each sample and a BioRad molecular weight standard marker were run on a 10% SDS-PAGE, followed by transfer to nitrocellulose membranes. Nonspecific binding to the membranes was blocked with 10% nonfat milk. Primary polyclonal (rabbit) antibodies and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were used in conjunction with the ECL Chemiluminescence Kit (Amersham Pharmacia Biotech, NJ) and membranes were exposed to radiographic films for detection. Western blots were digitized for comparison of the intensity for each band using the ImageTool image analysis software program (University of Texas Health Science Center, San Antonio, TX). The integrated density of each band was measured using identical 1480 pixel areas of each Apfa-1 or Actin band at a scale of densities from 0 to 255. The ratios of the integrated densities for Apfa-1 /Actin are compared for mammary epithelial cells in Figure 1A, and keratinocytes in Figure 2A.

Caspase 3 activity assay: The Caspase 3 Apoptosis Detection Kit was purchased from Santa Cruz Biotech. Inc. In a 24 well tissue culture plate, 10^5 cells/well of control or treated cells in triplicates were plated. After 24 hr treatments with EGCG and GTPPs, the cells in each well were washed with 1 ml PBS and incubated with 100 µl lysis buffer on ice for 10 min. To each well, 100 µl of 2X reaction buffer was added with 10 mM DTT. Finally, 5 µl of DEVD-AFC substrate was added to each well containing cell lysates. The reaction mixtures were incubated for 1 hour at 37o C. The caspase 3 activity in each well was measured using a fluorescence plate reader set for 405 nm excitation and 505 nm emission.

RESULTS

The mammary epithelial cells maintained basal levels of p57 protein regardless of EGCG exposure (Fig. 1A). In contrast, protein levels of Apaf-1 were increased in conjunction with increased EGCG concentrations. Densitometry measurement demonstrated that the Apaf-1 protein levels were increased from 47% to 260% above control when EGCG concentration increased from 15 to 200 μM, while no significant changes were found in p57 levels (Fig. 1A). The epidermal keratinocytes previously have been characterized for their response to EGCG or GTPPs resulting in p57 induction without apoptosis (1, 21). In response to increasing concentrations of EGCG, these cells expressed stable basal levels of Apaf-1 and consistent high levels of PCNA (Fig, 2A).

The mammary epithelial cells responded to EGCG by a linear elevation of caspase 3 activities with the exception of 200 μM EGCG (Fig. 1B.). The keratinocytes, however, only exhibited basal levels of caspase 3 activities (Fig. 2B.). The mammary epithelial cells showed little change in morphology 24 h after incubation with 50 μM EGCG (Fig. 3B), in comparison to the control cells (Fig. 3A). Significant cell death was observed after 48 h treatment with 50 μM EGCG (Fig. 3D) compared to 48 h control cells (Fig. 3C). Morphological changes were seen as alterations in cell shape as well as cell blebbing. In addition, many cells appeared to be flattened, and the occupied space was still less than that observed in the untreated controls. At 96 h, these characteristics were more apparent (Fig. 3F) compared to the control, which became a confluent monolayer (Fig. 3E).

In 0.2 µg/ml GTPPs for 48 h, both p57-transfected OSC2 clones demonstrated significant resistance to GTPPs-induced apoptosis (Fig. 4A and 4C). The trypan blue staining was noted only in the superficial stratum of cells, with a large number of living cells attached. The p57 antisense-transfected clones did not survive the GTPPs exposure. In fact, all cells were lysed or stained with trypan blue (Fig. 4B and 4D). The green fluorescent protein (GFP)-transfected clone, as an internal control, showed identical apoptosis to the parental cells (1) by cell lysis with diminished green fluorescence (Fig. 4F), while the untreated controls exhibited bright green fluorescence (Fig. 4E).

DISCUSSION

Unlike the keratinocytes, mammary epithelial cells under in vivo conditions could not be exposed to EGCG concentrations higher than 4.4 µM, the maximum human plasma concentration (22). Therefore, concentrations higher than that could be potentially damaging to mammary epithelial cells, which has been proven to be true in this study. The fundamental difference in response to EGCG between mammary epithelial cells and the epidermal keratinocytes is that p57 induction-associated cell survival is only present in the keratinocytes. In the mammary epithelial cells, while p57 protein levels remained unchanged, Apaf-1 levels increased as high as 260% in response to increasing concentrations of EGCG. In addition, increased caspase 3 activities paralleled increased EGCG concentration; 100 µM EGCG induced a 3-fold increase in caspase 3 activity at 24 h compared to control. We suspect that lowered caspase 3 activity in 200 µM EGCG was due to a plateau of the caspase 3 activity. The mammary epithelial cells have a higher background in caspase 3 activity than the keratinocytes.

This may be due to a larger cell population undergoing apoptosis constitutively in mammary epithelial cells compared to the keratinocytes.

Apaf-1 accumulation, caspase 3-activation, and cell detachment/shrinkage and blebbing often are observed in mitochondrion-mediated apoptosis, and these characteristics are exhibited by EGCG-treated mammary epithelial cells. These results indicate that absence of p57 response to EGCG may lead to mitochondrion-mediated apoptosis even in normal cells. In addition, these results also suggest that EGCG concentrations higher than the maximum plasma concentration may be applied only topically (including oral application). In contrast, the normal epidermal keratinocytes showed constant basal levels of Apaf-1 regardless of time or dose of EGCG treatment, indicating a mechanism resisting apoptosis. During development, p57 and Apaf-1 may work collaboratively since the only cyclin dependent kinase inhibitor essential to development is p57 (23), and Apaf-1 also is actively involved (16). The survival/death linkage between p57 and Apaf-1 through the Rb/E2F pathway also may play an important role in regulation of differentiation and apoptosis in epidermal epithelial cells.

Based on the evidence that; 1) normal human keratinocytes in growth arrest are resistant to apoptosis (24), 2) GTPPs/EGCG failed to induce apoptosis in the keratinocytes (1, 21, 25), and 3) p57 induces G1 growth arrest and differentiation (6, 12), it is evident that p57-induction protects the human epithelium from green tea-induced apoptosis, possibly through growth arrest and/or differentiation, and cells failing to elevate p57 would enter a mitochondrion-mediated, caspase 3-dependent apoptotic pathway.

The role of p57 in resisting GTPPs-induced apoptosis is further demonstrated by the survival of the metastatic oral squamous cell carcinoma OSC2 cells transfected with p57 sense cDNA. None of the parental OSC2 cells (1, 21), p57 antisense-transfected, and green fluorescent protein (GFP)-transfected cells survived in 0.2 mg/ml GTPPs. This concentration is not higher than that in green tea drink preparations (26) but is lethal to many tumor cell lines. Only the p57 sense cDNA transfected OSC2 cells survived in this GTPPs concentration.

In conclusion, these data suggest that p57 plays a crucial and determinant role in cell survival during GTPPs or EGCG challenge. The differential effects of green tea on normal/tumor cells/tissues should be further investigated since future trial therapies would expose normal and tumor cells simultaneously to green tea polyphenols. Therefore, the p57-mediated survival pathway in normal epithelial cells where apoptosis is inhibited by GTPPs should be explored to pinpoint which apoptotic event is disrupted by p57 induction. However, how GTPPs or EGCG initiate apoptotic pathway(s) is not fully understood, as well as what mechanism GTPPs or EGCG activate to trigger the mitochondrial membrane permeabilization, leading to the release of apoptotic associated mediators such as cytochrome c. These parameters are currently being investigated by our group and also by other laboratories.

FIGURE LEGENDS

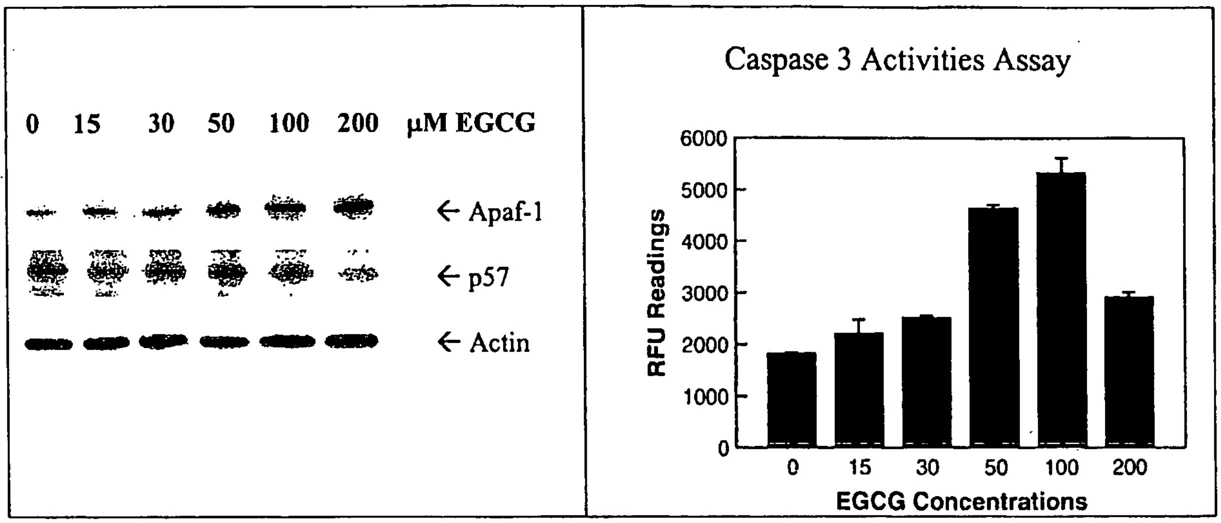
Figure 1. A: Western blot of whole cell lysates from mammary epithelial cells exhibiting up-regulation of Apaf-1 levels and basal p57 levels when treated with increasing concentrations of EGCG. B: caspase 3 activity assay performed on the same cells. Detection of caspase 3 activities was based on PARP cleavage by caspase 3. EGCG concentration ranged from 0 to 200 μM. Experiments were repeated three times with similar results. Each bar represents average of triplicate samples and SD.

Figure 2. A: Western blot of whole cell lysates from human epidermal epithelial cells with EGCG treatments as indicated. No significant changes shown in Apaf-1 bands or PCNA

bands measure by densitometry, compared to actin levels. B: caspase 3 activity assay performed on the same cells. No elevation of caspase 3 activity was recorded. EGCG concentration ranged from 0 to 200 μM. G: 0.2 mg/ml GTPPs. Experiments were repeated three times with similar results. Each bar represents average of triplicate samples and SD.

Figure 3. Normal human mammary epithelial cells were grown in MEGM without treatment for 24 h (A), 48 h (C), and 96 h (E). Under identical conditions, cells were treated with 50 μM EGCG for 24 h (B), 48 h (D), and 96 h (F). Original magnification: 400X.

Figure 4. OSC2 subclones S2, S3 (A and C, both transfected with p57 sense cDNA), A5, A3 (B and D, both transfected with p57 antisense cDNA) were treated with 0.2 mg/ml GTPPs for 48 h, changed to regular medium for additional 24 h, and then stained with trypan blue and photographed. OSC2 subclone Green6, transfected with GFP, without treatment (E) and treated with 0.2 mg/ml GTP for 48 hr, then placed in regular medium for another 48 hr (F). Original magnification: 400X.


0
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μM EGCG
← Apaf-1
← p57
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Caspase 3 Activities Assay
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6000
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3000
2000
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15
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EGCG Concentrations

Figure 1.

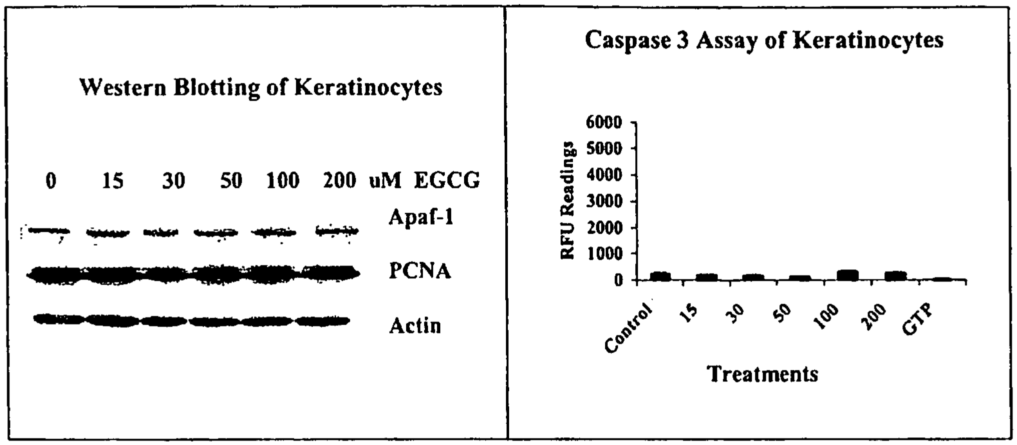

Fig.2

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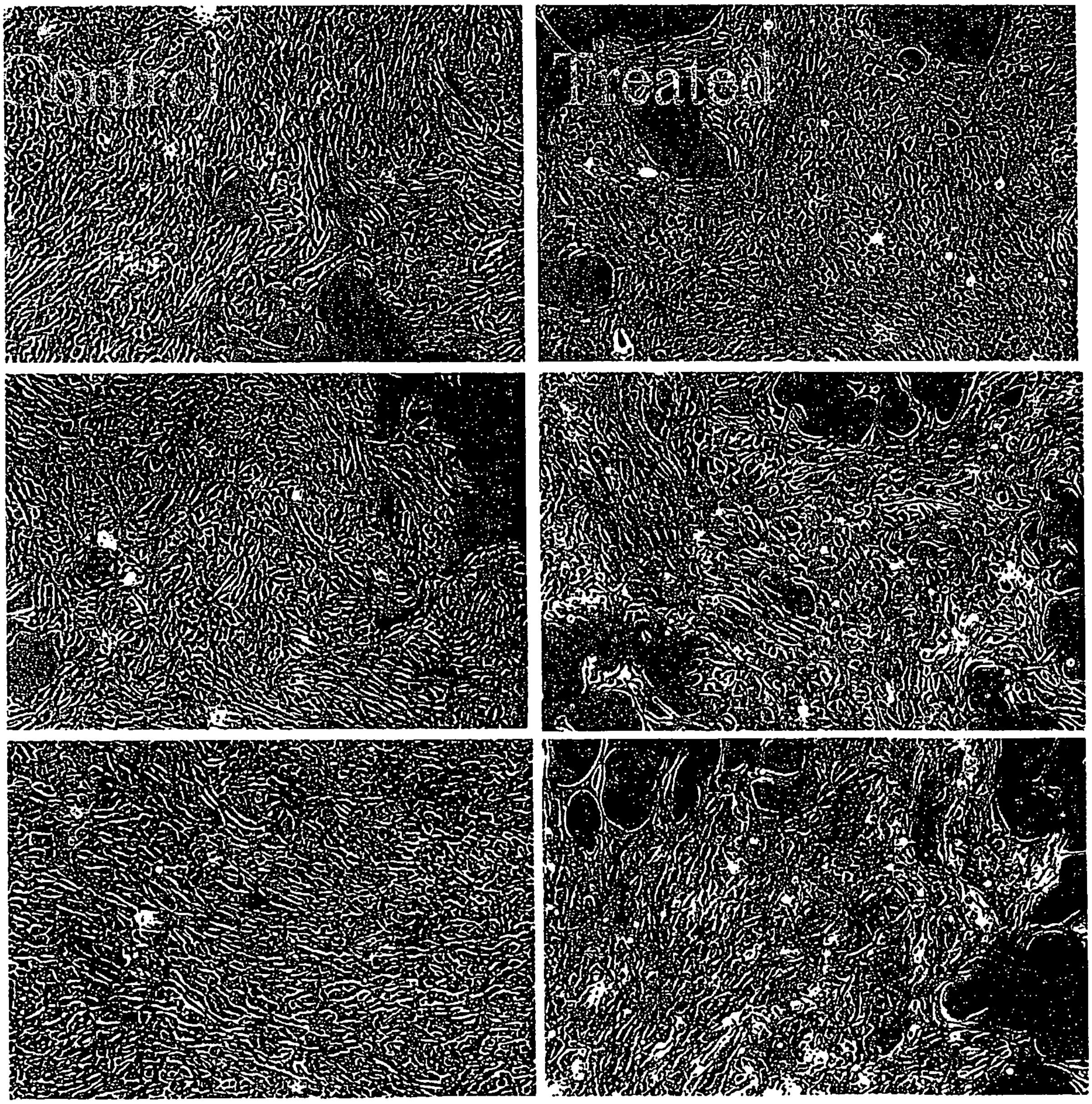
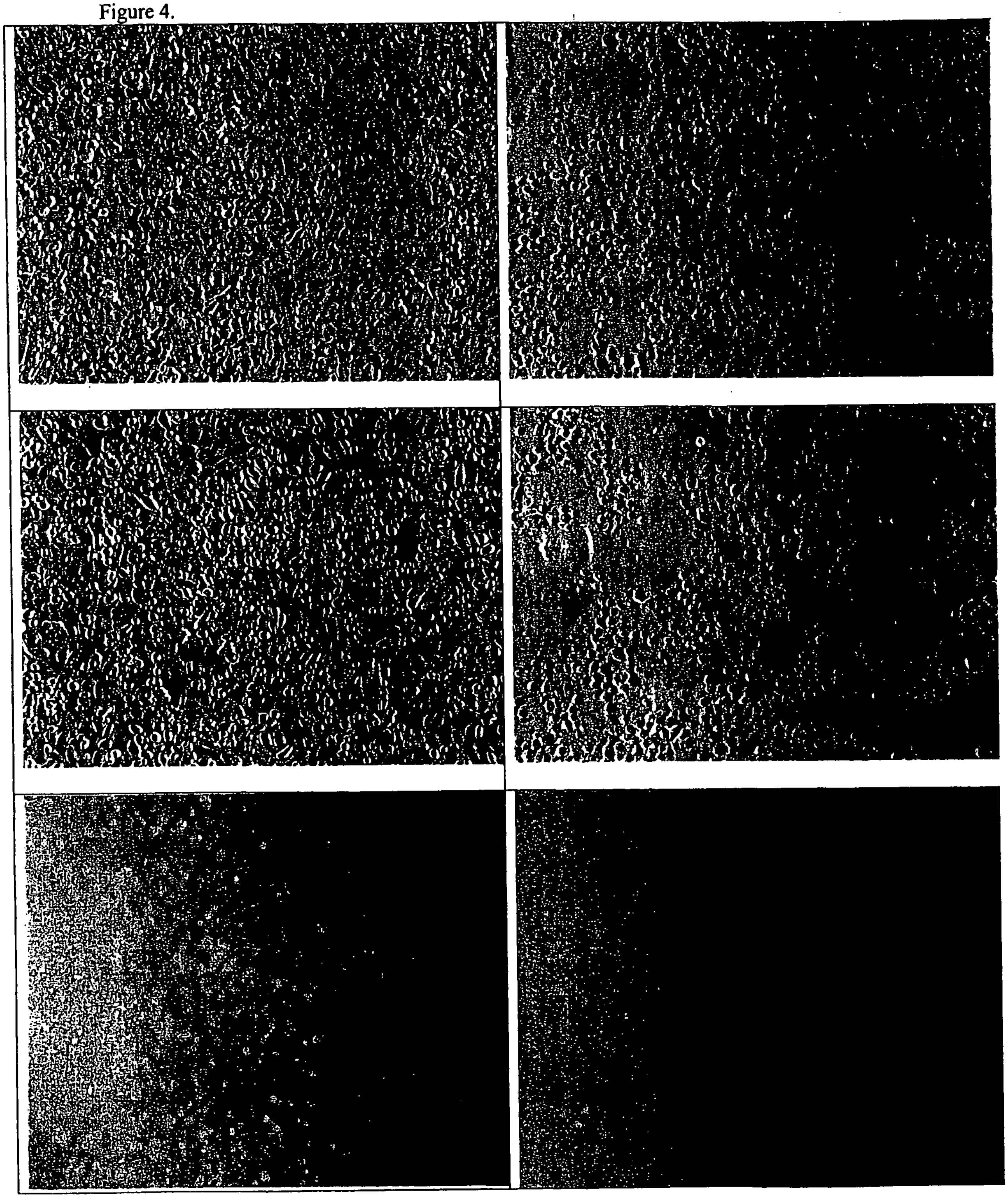


Figure 4.



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Example 4

Green Tea Polyphenol Targets the Mitochondria in Tumor Cells Inducing Caspase 3 Dependent Apoptosis

Induction of apoptosis by green tea polyphenols has been observed in various tumor cell systems, but whether green tea polyphenol-induced apoptosis requires caspase 3 for execution has not been confirmed. We previously reported that green tea polyphenol-induced apoptosis involved Apaf-1 accumulation and caspase 3 activation in the cytosol. In the current study, tumor cells either with deleted caspase 3 gene or expressing wild type caspase 3 were treated by increasing concentrations of green tea polyphenol(s), followed by morphological analysis and caspase 3 activity assay. The caspase 3 null parental cell line was further examined in comparison with a well-characterized, caspase 3 wild type oral carcinoma cell line by MTT assay and BrdU incorporation assay. The results demonstrated that while the mitochondrial function was gradually declined to insignificant levels, caspase 3 null cells did not undergo apoptosis, which suggested that green tea polyphenol-induced apoptosis is a mitochondria-targeted, caspase 3 executed mechanism.

One of the chemopreventive effects of green tea polyphenols has been recognized for their ability to induce growth arrest and/or cell death in certain tumor cell types (1-5). The four major polyphenols present in green tea are (-)-Epicatechin (EC), (-)-Epigallocatechin (EGC), (-)-Epcatechin-3-gallate (ECG) and (-)-Epigallocatechin-3-gallate (EGCG) (collectively referred as GTPPs hereafter). Reports from our group and others have indicated that caspase 3 plays an important role for GTPPs-induced apoptosis in human cancer cells (6-9). We have demonstrated that using concentrations lower than the concentrations in green tea drink preparations (300-600 μM for EGCG, 0.38-0.76 g/ml for the four major polyphenols), GTPPs or EGCG alone were able to induce apoptosis in oral squamous carcinoma cells, while normal human epidermal keratinocytes survived (10, 11). We observed that EGCG-induced apoptosis involves Apaf-1 and caspase 3, two key factors in the mitochondria-mediated apoptosis pathway (11). However, whether caspase 3 plays a determinant role is unknown; since other apoptotic pathways might be involved, e.g. TNF alpha or Fas induced-death receptor pathway and autophagy pathway (12). Elucidation of GTPPs-induced specific apoptosis pathway is crucial to future chemopreventive or therapeutic intervention designs utilizing GTPPs, since certain tumor cells may be resistant to GTPPs. To examine the role of caspase 3 in GTPPs-induced apoptosis, we took advantage of MCF7 (caspase 3 null) cells, which are resistant to caspase 3-executed apoptosis but are able to execute caspase 3-independent apoptosis (13-15). The current investigation was designed to test our hypothesis that GTPPs specifically induce a caspase 3-dependent apoptosis pathway, initiated by mitochondrial cytochrome c release in tumor cells.

The tumor cells selected for this investigation either express wild-type caspase 3 (OSC2, MCF7 caspase 3 +), or are caspase 3 null (MCF7), for determining whether caspase 3 is essential in EGCG/GTPPs-induced apoptosis. The OSC2 cell line was isolated from submandibular lymph node metastasis of a 68-year old female, the primary tumor being located in the gingiva of this patient (16). OSC2 cells have a p53 mutation at exon 8, site 280, resulting in Arg to Thr substitution (17) and over-expression of p53 (18). The doubling time for OSC2 cells is 22.9 h. MCF7 cells were obtained from American Type Culture Collection (HTB22). MCF7 cells are defective in caspase 3-executed apoptosis and show a lack of downstream events, e.g. DNA fragmentation, cellular shrinkage, and blebbing, due to a deletion in the caspase 3 gene (19). MCF7 caspase 3 + cells were generated by stable caspase 3 cDNA transfection of MCF-7 cells.

The defective functions described above were restored in these cells (20). A concentration gradient of EGCG and 0.2 mg/ml GTPPs was tested for the apoptotic effect in the three tumor cell lines. Pooled human neonatal epidermal keratinocytes were used as negative control for caspase 3 activation. These normal cells are able to survive in GTPPs through a p57 mediated pathway described previously (10, 11).

MATERIALS AND METHODS

Chemicals: EGCG was purchased from Sigma (St. Louis, MO). A mixture of four major GTPPs was purchased from LKT Lab. Inc (Minneapolis, MN). GTPPs and EGCG were dissolved in cell culture medium and filter-sterilized immediately prior to use. 50 µl of EGCG equals 22.9 µg/ml.

Cell lines and cell culture: The normal human keratinocytes (NHEK CC-2507) were purchased from Cambrex (East Rutherford, NJ) and maintained in KGM-2 medium (Cambrex). The OSC2 cell line was previously described (16). The breast carcinoma MCF7 cell line was purchased from American Type Culture Collection (ATCC). MCF7(C) caspase + cell line "7-3-28" was established and tested as previously described (19, 20). These tumor cells were maintained in 45% Dulbecco's Modified Eagle's Medium (DMEM), 45% Ham's F12 medium and 10% fetal calf serum, 100 Unit/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml hydrocortisone. All cell cultures were maintained in a 37o C incubator with 5% CO_2. Light microscopic photographs were taken with a SPOT RT digital camera system (Diagnostic Instruments) linked to a Nikon Phase Contrast-2 microscope at an original magnification of 200X.

Caspase 3 activity assay: The Caspase 3 Apoptosis Detection Kit was purchased from Santa Cruz Biotech. Inc. In a 24 well tissue culture plate, 10^5 cells/well of cells in triplicates were plated. After 24 hr treatments with EGCG and GTPPs, the cells in each well were washed with 1 ml PBS and incubated with 100 ml lysis buffer on ice for 10 min. To each well, 100 µl of 2X reaction buffer was added with 10 mM DTT. Finally, 5 µl of DEVD-AFC substrate was added to each well containing cell lysates. The reaction mixtures were incubated for 1 hour at 37o C. The caspase 3 activity in each well was measured using a fluorescence microplate reader set for 405 nm excitation and 505 nm emission.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay: This method detects the activity of mitochondrial succinate dehydrogenase (SDH). In a 96-well plate, $1.5X10^4$ cells were seeded in each well. After variety of treatments, 100 µL of 2% MTT was added to each well and the plate was incubated at 37o C for 30 minutes. 100 µl of 0.2 M Tris (pH 7.7) with 4% Formalin was added to each well. After incubation at room temperature for 5 minutes, liquid was removed and the wells were allowed to dry. Each well was rinsed with 200 µl water followed by addition of 100 µl DMSO (6.35 % 0.1 N NaOH in DMSO) to each well. The coloration was measured by a Thermo MAX microplate reader (Molecular Devices Corp. Sunnyvale, CA) using wavelengths of 562 nm. Experiments were repeated three times with triplicate samples for each experiment.

DNA synthesis analysis using BrdU incorporation method: The BrdU cell proliferation kit was purchased from Oncogene Research Products, Boston, MA. Cells were culture in 96 well plates with 10^4 cells/well. After EGCG and GTPPs treatments, cells were labeled by BrdU, reacted with BrdU antibody and the color reaction was carried out according to the protocol provided by the manufacturer. The coloration was measure by a Thermo MAX microplate reader using wavelengths 450 nm – 562 nm. Experiments were repeated three times with triplicate samples for each experiment.

Cell growth assay: MCF7 cells were seeded (5X104) in 25 cm^2 tissue culture flask for 24 h prior to EGCG or GTPPs incubation. Cells from each flask were trypsinized and counted at each time point on a hemacytometer. Results are based on three repeated experiments.

RESULTS

Morphological analysis: The light microscopy photos in Figure 1 indicated that both EGCG and GTPPs induced apoptosis only in caspase 3 + MCF7(C) cells (Fig. 1D and 1F). MCF7(C) cells exhibit differential morphology when compared with MCF7 cells (Fig. 1A and 1B). EGCG treatment for 48 h significantly reduced the cell number and produced cell blebbing (Fig. 1D), a characteristic of caspase 3 dependent apoptosis (20). After MCF7(C) cells were exposed to 0.2 mg/ml GTPPs for 24 h, the majority of the cells were either exhibited apoptosis or became fragmented (Fig. 1F). When incubation with 0.2 mg/ml GTPPs extended to 48 h, no viable MCF7(C) cells remained (data not shown). In contrast, caspase 3 null MCF7 cells did not exhibit reduction in cell density nor cell death after 24 h GTPPs treatment, while increased cell density was observed in 48 h EGCG treated cells (Fig. 1C) compared to 24 h control cultures (Fig. 1A), indicating that cell growth was not inhibited during EGCG treatment.

Caspase 3 activity assay: When increasing concentrations of EGCG and 0.2 mg/ml GTPPs were incubated with MCF7 and MCF7(C) cells, MCF7(C) demonstrated activated caspase 3 detected by PARP cleavage-based caspase 3 activity assay (Fig. 2A). The pattern of caspase 3 activation was very similar to that of OSC2 cells, which served as a positive control (Fig. 2C). Both OSC2 cells and MCF7(C) cells were efficiently induced to apoptosis by GTPPs in 24 h in morphological analysis (10, 11 and Fig.1F). On the contrary, MCF7 cells did not show caspase 3 activity (Fig. 2B) compared to the normal human epidermal keratinocytes (Fig. 2D), which served as a negative control and was protected by a p57/KIP2-mediated survival pathway (9, 21).

BrdU assay: OSC2 cells were used in the BrdU incorporation assay as positive growth inhibition control (Fig. 3). OSC2 cells ceased BrdU incorporation when EGCG concentrations reached 50 μM (Fig 3A), while MCF7 cells were able in incorporate BrdU efficiently except in 0.2 μg/ml, where the incorporation decreased, but was not diminished (Fig.3B).

Cell growth assay and MTT assay: Continued culturing of MCF7 cells in 50 μM EGCG for 96 h showed only an insignificant decrease in cell number compared to untreated cultures (Fig. 4A). However, mitochondrial SDH activities were significantly decreased when MCF7 cells were treated with 50 mM EGCG (Fig. 4B). When MCF7 cells were cultured in the presence of 0.2 mg/ml GTPPs, the SDH activities were completely diminished at 48 h time point (Fig. 4B).

The SDH activities in OSC2 cells decreased during a 24 h period when exposed to increasing concentrations of EGCG and 0.2 mg/ml GTPPs (Fig. 5A). The caspase 3 null MCF7 cells showed similar patterns when identical treatment was applied (Fig. 5B).

DISCUSSION

Previous reports have indicated that tea polyphenols induced apoptosis in various tumor cell types, associated with caspase 3 activation. In this regards, EGCG induced caspase 3 dependent apoptosis in human chondrocarcoma cells (8). Oolong tea (a form of semi-fermented tea in which polyphenols are partially preserved) polyphenol theasinensin A induced apoptosis in the human histocytic lymphoma cell line U937 through cytochrome c release and activation of caspase-9 and caspase-3 (21). Cytochrome c release and caspase activation were also observed in Ehrlich ascites tumor cells when exposed to green tea extract (22). These data suggested that GTPPs-induced apoptosis in cancer cells correlated with cytochrome c release and caspase 3 activation.

The current study was designed to address three key questions: 1) whether wild type caspase 3 is required for GTPPs-induced apoptosis; 2) how normal human epithelial cells respond to the GTPPs treatment in terms of caspase 3 activation, and 3) whether GTPPs diminishes the mitochondrial activity in the absence of wild type caspase 3. Results shown in Fig. 1 indicated that caspase 3 is a determinant factor for GTPPs-induced apoptosis. GTPPs at the concentration of 0.2 mg/ml was able to eliminate the majority of MCF7(C) cells in 24 h (Fig. 1F), while the parental caspase 3 null MCF7 cells did not exhibit any morphological alterations (Fig.1E). Similar patterns were observed when 50 µM EGCG was applied for 48 h (Fig.1C and 1D). Lack of wild type caspase 3 in MCF7 cells maintained their survival due to lack of caspase 3 activity (Fig.2B), while caspase 3 + MCF7(C) cells activated caspase 3 as much as 9 fold (Fig. 2A), which correlated with apoptotic morphology (Fig. 1F). The caspase 3 activities of MCF7 and MCF7(C) cells were verified by using OSC2 cells as a positive control (Fig. 2C); and human epidermal keratinocytes as a negative control (Fig. 2D). These results strongly suggest that wild type caspase 3 is the executer for GTPPs-induced apoptosis; therefore absence of any element in caspase 3 dependent apoptosis pathway could result in resistance to GTPP-induced apoptosis. The caspase 3 null MCF7 cells were not only resistant to GTPP-induced apoptosis, but also demonstrated continued growth in the presence of 50 µM EGCG for up to 96 h (Fig.1C and Fig.4A). These results, in addition to data from the BrdU assay (Fig.3B), suggest that EGCG is not able to induce growth arrest in MCF7 cells giving the fact that MCF7 cells possesses wild type p53, and the TGF beta and insulin signaling pathways are intact (23-25). The interesting finding is that while MCF7 cells survived GTPPs/EGCG exposure and continue to proliferate, the mitochondria function was gradually impaired by either EGCG or GTPPs initiated at 24 h (Fig.4B), and completely depleted by GTPPs in 48 h (Fig.5B). We postulate that EGCG at 50 μM concentration is not able to completely eliminate the mitochondrial function (Fig.5B); the energy supply for cell proliferation could be provided for the period up to 96 h. Data from this study and previous investigations indicate that green tea polyphenols target the mitochondria, leading to cytochrome c release and apoptosome formation, and subsequently activate the caspase 3 dependent apoptosis pathway. Cancer cells lacking wild type caspase 3 may be resistant to GTPPs to undergo immediate apoptosis, but the mitochondria could be damaged in a prolonged time period. We have reported that p57 is a determinant factor for cells survival during GTPPs treatment using either p57 inducible human epidermal keratinocytes or retroviral-transfected OSC2 cells expressing wild type p57 (11). However, how mitochondria of the keratinocytes are protected during the activation of this survival pathway is unknown, as well as how GTPPs inflict mitochondrial membrane permeabilization in the cells without the protective mechanism. These issues will be addressed in future investigations to understand how GTPPs selectively activate specific pathways, leading to opposite consequences that could be beneficial to potential chemoprevention or therapeutic interventions against cancer.

LEGENDS OF FIGURES

Figure 1. A. Caspase 3 null MCF7 cells were cultured for 24 hours without treatment. B. Caspase 3 + MCF7(C) cells were cultured in the same condition as in A.

C. MCF7 cells were cultured in the presence of 50 μM EGCG for 48 h. D. MCF7(C) cells were cultured in the same condition as in C. E. MCF7 cells were cultured in 0.2 mg/ml GTPPs for 24 h. F. MCF7(C) cells were cultured in the same condition as in E. Only MCF7(C) cells exhibited apoptotic morphology when exposed to either EGCG or GTPPs.

Figure 2. Caspase 3 activity assay results showing elevated caspase 3 activities in MCF7(C) cells (A) in comparison with MCF7 cells (B). MCF7(C) cells responded to increasing concentrations of EGCG and 0.2 mg/ml GTPPs in a 24 h period similarly to OSC2 cells (C), a well-characterized oral squamous cell carcinoma cell line that undergoes apoptosis when exposed to GTPPs. Both cell lines exhibited highest levels of caspase 3 activities in response to 0.2 mg/ml GTPPs. The caspase 3 null MCF7 cells responded to identical treatment similarly to normal human epidermal keratinocytes, which also failed to elevate caspase 3 activities (D). Experiments were repeated three times. Each column represents the average of triplicate samples and SD.

Figure 3. BrdU incorporation assay results showing OSC2 oral carcinoma cells ceased BrdU incorporation when exposed to EGCG concentrations greater than 50 μM or GTPPs (GTP) (A). Under identical conditions, the caspase 3 null MCF7 cells exhibited normal levels of BrdU incorporation compared to control with sight decrease when exposed to GTPPs (B). Experiments were repeated for three times with similar patterns. Each column represents the average of triplicate samples and SD.

Figure 4. A: Caspase 3 null MCF7 cells did not show significant growth inhibition by 50 μM EGCG when the cells were cultured for the indicated time periods. Each column represents the average of triplicate samples and SD. B: MCF7 cells showed significant loss in

[redacted]

mitochondrial SDH activities when treated with 50 μM EGCG or 0.2 mg/ml GTP for the indicated time periods. Each column represents the average of triplicate samples and SD.

Figure 5. MTT assays results showing decreasing mitochondrial SDH activities are associated with increasing concentrations of EGCG as indicated or 0.2 mg/ml GTPPs (GTP) in both OSC2 cells (A) and caspase 3 null MCF7 cells (B). Both cell lines exhibited lowest SDH activities when exposed 0.2 mg/ml GTPPs for 24 h. Experiments were repeated three times with similar patterns. Each column represents the average of triplicate samples and SD.

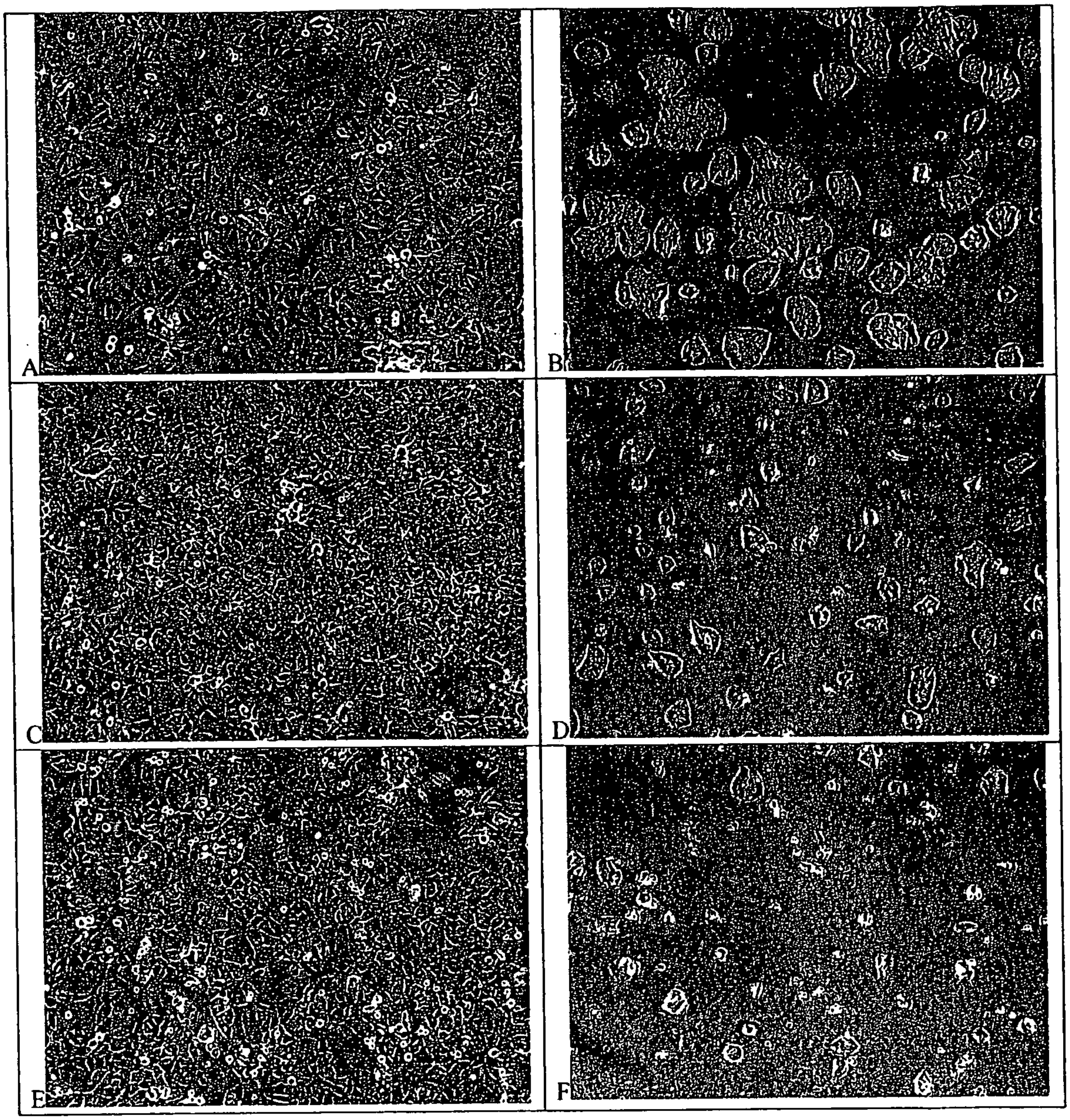

Figure 1

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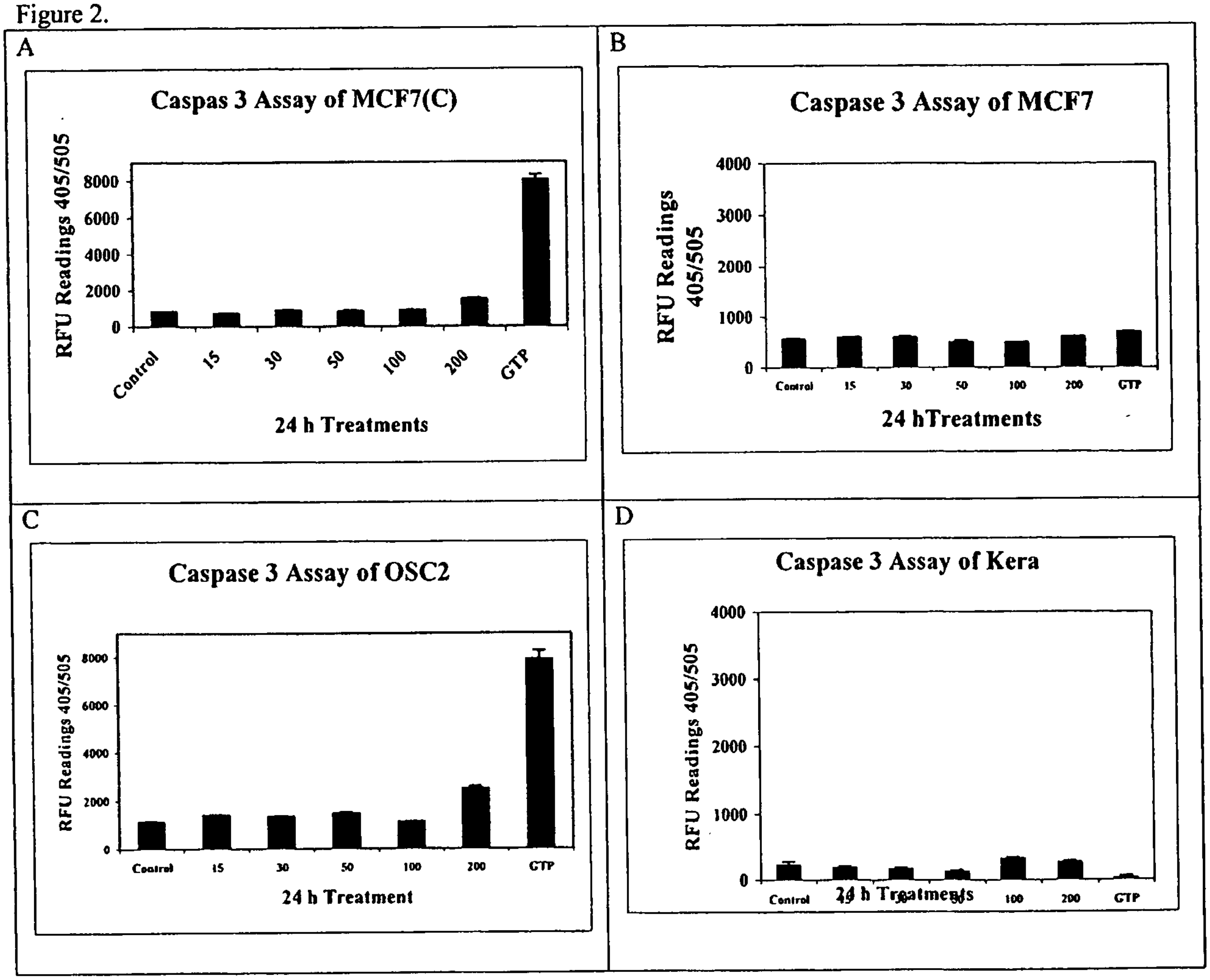
Figure 2.


A
Caspas 3 Assay of MCF7(C)
RFU Readings 405/505
8000
6000
4000
2000
0
Control
15
30
50
100
200
GTP
24 h Treatments
B
Caspase 3 Assay of MCF7
RFU Readings 405/505
4000
3000
2000
1000
0
Control
15
30
50
100
200
GTP
24 hTreatments
C
Caspase 3 Assay of OSC2
RFU Readings 405/505
8000
6000
4000
2000
0
Control
15
30
50
100
200
GTP
24 h Treatment
D
Caspase 3 Assay of Kera
RFU Readings 405/505
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Control
15
30
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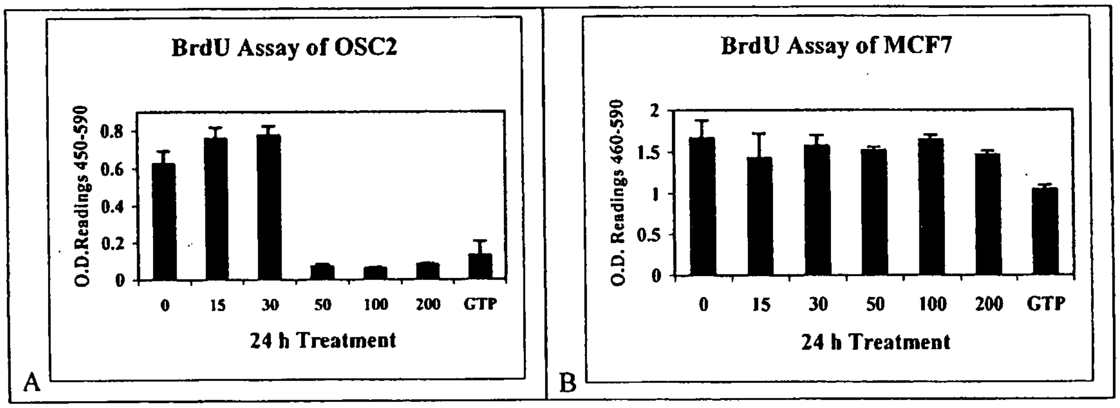
200
GTP
24 h Treatments


A
BrdU Assay of OSC2
O.D.Readings 450-590
0.8
0.6
0.4
0.2
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0
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30
50
100
200
GTP
24 h Treatment
B
BrdU Assay of MCF7
O.D. Readings 460-590
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1.5
1
0.5
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15
30
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200
GTP
24 h Treatment

Figure 3.

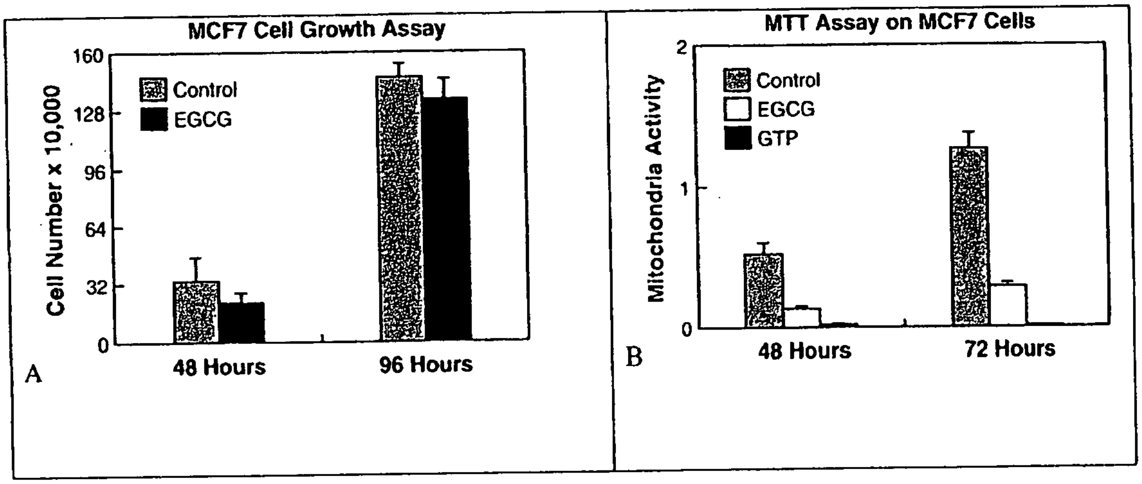
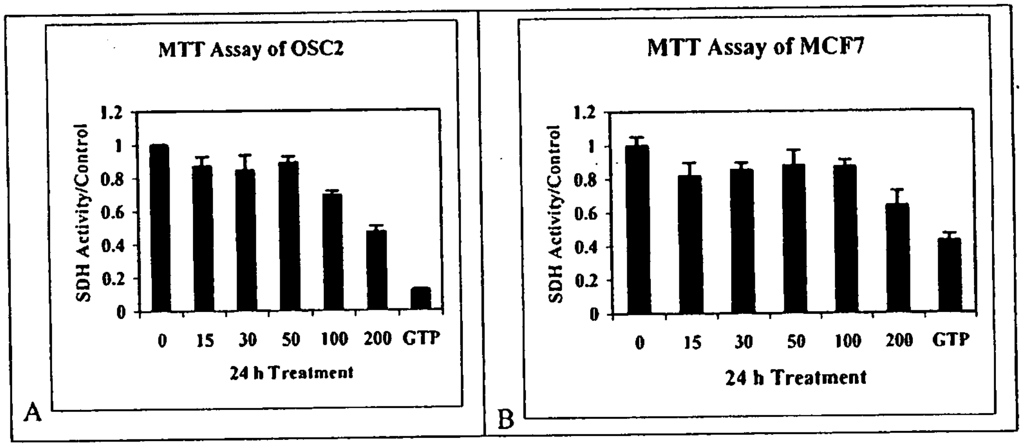

MCF7 Cell Growth Assay
Cell Number x 10,000
160
128
96
64
32
0
▨ Control
■ EGCG
48 Hours
96 Hours
A
MTT Assay on MCF7 Cells
Mitochondria Activity
2
1
0
▨ Control
☐ EGCG
■ GTP
48 Hours
72 Hours
B

Figure 4


MTT Assay of OSC2
SDH Activity/Control
1.2
1
0.8
0.6
0.4
0.2
0
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15
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200
GTP
24 h Treatment
A
MTT Assay of MCF7
SDH Activity/Control
1.2
1
0.8
0.6
0.4
0.2
0
0
15
30
50
100
200
GTP
24 h Treatment
B

Figure 5.

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Example 5

Tea Polyphenols Induce Differentiation and Proliferation in Epidermal Keratinocytes

The most potent green tea polyphenol, epigallocatechin-3-gallate (EGCG), was found to induce differential effects between tumor cells and normal cells. Nevertheness, how normal epithelial cells respond to the polyphenol at concentrations for which tumor cells undergo apoptosis is undefined. The current study tested exponentially growing and aged primary human epidermal keratinocytes in response to EGCG or a mixture of the four major green tea polyphenols. EGCG elicited cell differentiation with associated induction of p57/KIP2 within 24 hours in growing keratinocytes, measured by the expression of keratin 1, filaggrin and transglutaminase activity. Aged keratinocytes, which exhibited low basal cellular activities after culturing in growth medium for up to 25 days, renewed DNA synthesis and accelerated energy production up to 37-fold upon exposure to either EGCG or the polyphenols. These results suggest that tea polyphenols may be used for treatment of wounds or certain skin conditions characterized by altered cellular characteristics.

Green tea polyphenols (referred to as GTPPs, to include the four major polyphenols [-]-Epicatechin [EC], [-]-Epigallocatechin [EGC], [-]-Epcatechin-3-gallate [ECG] and [-]-Epigallocatechin-3-gallate [EGCG]) have been identified to possess chemopreventive and apoptotic activity against certain cancers, while normal epidermal keratinocytes follow a survival pathway that has not been fully elucidated [1-10]. The most abundant green tea polyphenol, epigallocatechin-3-gallate (EGCG), was reported to induce differentiation and growth arrest in epidermal keratinocytes [11], rather than apoptosis as it does in tumor cells [3-5, 10].

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We previously reported that both GTPPs and EGCG were able to induce transient expression of p57/KIP2, a differentiation/cell cycle regulator, which was associated with cell survival during GTPP exposure [3]. It was proposed that p57 induction may stimulate cell differentiation as part of a survival pathway [3, 5, 6]. While this survival pathway is currently under investigation, the impact of GTPPs on epidermal keratinocytes located in various layers of the skin was deemed essential to be addressed, given the fact that GTPPs are able to penetrate the epidermis, but not the dermis, of human skin [12].

Keratinocytes within the epidermis exist in various stages of differentiation corresponding to different epidermal layers [reviewed in 13]. For example, the basal keratinocytes and/or stem cells at the dermal-epidermal junction continuously proliferate to regenerate and restore cells lost to the environment. As the daughter cells migrate up through the epidermal layers, they first undergo growth arrest followed by expression of keratins 1 and 10 in the spinous layer. In the next layer, the granular layer, late markers of keratinocyte differentiation, including filaggrin and other structural proteins, are expressed. In addition, the activity of transglutaminase, the enzyme that cross links the structural proteins into the cornified envelope, is increased. Finally, the keratinocytes undergo an epidermal-specific programmed cell death to form the cornified layer, which serves as a barrier to mechanical injury, microbial invasion and water loss. The entire epidermis turns over in one to two months, although the transit time of keratinocytes may be lengthened or shortened in various disease states. We considered it pertinent to investigate whether GTPPs induce differential effects among keratinocytes at different stages of differentiation and/or age, knowing that if so, such effects could be significant for assessing the potential impact of these compounds upon topical application. Thus, agents that accelerate growth and/or differentiation of epidermal keratinocytes may shorten the healing time of certain wounds and serve as treatments for conditions such as aphthous ulcers and other epidermal-skin diseases.

We hypothesized that green tea polyphenols, either in a mixture or in the form of purified EGCG, are able to increase cellular activities, including new DNA synthesis, in aged keratinocytes, or promote differentiation of exponentially growing keratinocytes located in the basal layer of epidermis. Therefore, the current study examined pooled normal human primary epidermal keratinocytes treated with EGCG or GTPPs after various times of culture. Results from this study demonstrated that: 1) by promoting ATP production and new DNA synthesis, both EGCG and GTPPs "re-energized" the aged keratinocytes; thus, these compounds can presumably stimulate the regeneration of keratinocytes in aging skin; 2) by induction of p57, keratin 1 and filaggrin expression, and activation of transglutaminase, EGCG also stimulated the differentiation of the keratinocytes found in the basal layer of the epidermis. The combination of these two effects may help to accelerate wound healing and regeneration of new skin tissue, and subsequently prevent scar tissue formation. In addition, certain epithelial conditions may be amenable to treatment by topical applications of green tea polyphenols.

MATERIAL AND METHODS

Chemicals and antibodies: EGCG was purchased from Sigma (St. Louis, MO). A mixture of four major green tea polyphenols (GTPPs) was purchased from LKT Lab, Inc (Minneapolis, MN). GTPPs and EGCG were dissolved in keratinocyte growth medium-2 (KGM-2, BioWhittaker) and filter-sterilized immediately prior to use. The rabbit anti-human p57 antibody C-19 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the rabbit anti-filaggrin and anti-keratin-1 antibodies were from Covance (Berkeley, CA).

Culturing normal human epithelial cells: The pooled normal human primary epidermal keratinocytes were purchased from BioWhittaker (East Rutherford, NJ) and sub-cultured in the specific growth media provided by the manufacturer (KGM-2). Subculture of the epithelial cells was performed by detaching the cells in 0.25% trypsin and transferring into new tissue culture flasks, at the recommended density of 3500 cells/cm2. Exponentially growing keratinocytes were treated and harvested in their early passages (2-3 passages). Aged keratinocytes were allowed to grow in 96-well tissue culture plates for 15, 20 and 25 days prior to treatment by EGCG or GTPPs, followed by various assays.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay: This method detects the activity of mitochondrial succinate dehydrogenase (SDH). In a 96-well plate, $1.5X10^4$ cells were seeded in each well. After 24 h treatment, culture medium was removed and replaced with 100 μL of 2% MTT in a solution of 0.05 M Tris, 0.5 mM MgCl2, 2.5 mM CoCl2, and 0.25 M disodium succinate (Sigma, St. Louis, MO) and the plate was incubated at 37o C for 30 minutes. Cells were fixed in situ by the addition of 100 ml of 4% formalin in 0.2 M Tris (pH 7.7), and after a 5 min incubation at room temperature liquid was removed and the wells were allowed to dry. Each well was rinsed with 200 μl water and cells were solubilized by the addition of 100 μl of 6.35 % 0.1 N NaOH in DMSO. The colored formazan product was measured by a Thermo MAX micro plate reader (Molecular Devices Corp. Sunnyvale, CA) at a wavelength of 562 nm. Experiments were repeated three times with triplicate samples for each experiment.

Analysis of DNA synthesis using the BrdU incorporation method: The BrdU cell proliferation kit was purchased from Oncogene Research Products (Boston, MA). Cells were cultured in 96-well plates at the density of 10^4 cells/well. After EGCG and GTPPs treatments, cells were labeled with BrdU for 12 hours and levels of BrdU incorporation determined according to the manufacturer's instructions using a Thermo MAX micro-plate reader at a wavelength of 450 nm and subtracting absorbance measured at 562 nm. Experiments were repeated three times in triplicate for each experiment.

Immunocytochemistry: Normal human keratinocytes were seeded in 8-well chamber slides (Nagle Nunc International, Naperville, IL) 12 h prior to EGCG treatment. At the end of a 24 h treatment, the slides were washed with PBS and fixed in a cold 4% paraformaldehyde solution for 10 min. Then 3% hydrogen peroxide solution and normal goat serum were applied to block endogenous peroxidase activity and non-specific binding. The primary antibodies, rabbit-anti-human p57 polyclonal antibody C-19, rabbit anti-human keratin 1 and filaggrin antibodies were applied for 1 h at 37°C at the dilutions recommended by the manufacturers. The Streptavidin detection technique (Biogenex, USA) was used with 3-amino-9-ethylcarbazole as chromogen. Negative control sections consisted of tissues treated with 1% diluted normal goat serum instead of primary antibody. Mayer's hematoxylin was used as a counter-stain.

Transglutaminase activity assay: Normal human epidermal keratinocytes in early passages (2-3) were allowed to grow in 6-well tissue culture plates prior to EGCG exposure. The cells were scraped in homogenization buffer (0.1 M Tris/acetate, pH 8.5, containing 0.2 mM EDTA, 20 μM AEBSF, 2 μg/mL aprotinin, 2 μM leupeptin and 1 μM pepstatin A), collected by centrifugation and subjected to one freeze-thaw cycle prior to lysis by sonication. Unlysed cells were pelleted by centrifugation and aliquots of the supernatant collected for the determination of transglutaminase activity and protein concentration. Protein quantities were determined using the BioRad Protein Assay with bovine serum albumin as standard.

Transglutaminase activity was measured as the incorporation of [3H] putrescine into dimethylated casein, as described previously [14].

Caspase 3 activity assay: The Caspase 3 Apoptosis Detection Kit was purchased from Santa Cruz Biotech. Inc. Cells (10^5 per well) were plated in triplicate in a 24-well tissue culture plate. After 24 hr treatments with EGCG or GTPPs, the cells in each well were washed with 1 ml PBS and incubated with 100 µl lysis buffer on ice for 10 min. To each well, 100 µl of 2X reaction buffer was added with 10 mM DTT. Finally, 5 µl of DEVD-AFC substrate was added to each well containing cell lysates. The reaction mixtures were incubated for 1 hour at 37o C, and caspase 3 activity in each well was measured using a fluorescence micro-plate reader at a wavelength of 405 nm for excitation and 505 nm for emission.

RESULTS AND DISCUSSION

Unlike a variety of tumor cell types tested, normal human epidermal keratinocytes were able to survive when exposed to EGCG or GTPPs [2, 3, 8, 21, 23, 24]. This survival ability may be due to a differential intracellular response when normal keratinocytes are exposed to EGCG or GTPPs. The mechanism of the survival pathway is under investigation, but it may involve regulation of pro-survival factors, cell cycle factors and/or cell differentiation factors at the transcriptional and/or translational level [3, 5, 6, 11, 15, 16, 17, 18,]. In addition, responses of aged keratinocytes may differ from those of exponentially growing keratinocytes.

The pooled primary human epidermal keratinocytes, after 15, 20, or 25 days in culture, gradually lost their ability to either generate ATP or divide. At these time points, EGCG or GTPPs were able to activate the mitochondrial enzyme succinate dehydrogenase (SDH), as measured by the MTT assay (Fig. 1A, 1C and 1E), up to 37 fold (25 days, Fig. 1E). The

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activation of this component of the tricarboxylic acid (TCA) cycle may provide biological energy and substrates for other responses such as new DNA synthesis. When aged human keratinocytes lost the ability to synthesize new DNA, especially after 20 + days in KGM-2, both EGCG and GTPPs were able to stimulate new DNA synthesis, as measured by BrdU incorporation assay (Fig. 1B, 1D and 1F), up to approximately 3 fold (25 days, Fig. 1F). To our knowledge, this represents the first observation that green tea components stimulate energy generation and DNA replication in aged epidermal keratinocytes. It was noted that for the aged keratinocytes at the 15-day and 20-day time points, lower concentrations of EGCG (15-50 µM) had a slight negative impact on BrdU incorporation (Fig. 1B and 1D). On the other hand, EGCG concentrations higher than 100 µM consistently induced both SDH activity and BrdU incorporation (Fig.1). Therefore, the age of the keratinocytes and the concentration of EGCG or GTPPs used are two key factors in terms of the effects of these agents on energy generation and DNA replication. Of interest is the relationship of aged cultures of keratinocytes to their differentiation status. Since human keratinocytes are prone to undergo growth arrest and to express differentiation markers upon attaining confluence [19], we predict that the response of keratinocytes in upper epidermal layers will mirror that of the aged keratinocytes. Thus, we propose that EGCG and the GTPPs will stimulate reentry into the cell cycle in the early differentiated (spinous) stratum of the skin.

Previous data showing either growth arrest or differentiation of keratinocytes were based on observations in exponentially growing cells for which EGCG enhanced the expression of involucrin and increased the conversion of undifferentiated keratinocytes into corneocytes with concomitant growth arrest [11]. The current study further confirmed that the undifferentiated keratinocytes were able to commit to differentiation upon EGCG treatment within a short period of time, accompanied by an elevation in the activity of transglutaminase, the enzyme that cross-links involucrin and other substrates to form the cornified envelope [20]. When exponentially growing pooled normal human primary epidermal keratinocytes were incubated with 50-100 μM EGCG, these cells underwent differentiation in 24 h, as measured by immunocytochemistry using antibodies against human p57/KIP2 (a differentiation/growth arrest inducer), keratin 1 (an early differentiation marker), filaggrin (a late differentiation marker) (Fig.3), and transglutaminase activity assay (a late differentiation marker) (Fig.2). Note also that exposure to EGCG induced an increase in the number of enlarged, flattened, squame-like cells observed in these cultures. This morphology is typical of differentiated keratinocytes, providing further confirmation of the ability of EGCG to trigger cell differentiation. EGCG concentrations of 50 -100 μM were adequate to induce cell differentiation and were accompanied by a marked p57 elevation (Fig. 3B), indicating p57 may not only be responsible for cell survival but also for cell differentiation [3, 5, 6]. The EGCG concentrations used are within the physiological range in humans [18, 21, 22, 23, 24 and 25], given the fact that after drinking preparations equivalent to two to three cups of green tea, EGCG secreted from human saliva, excluding other polyphenols, was measured at concentrations up to approximately 50 μM (22.9 μg/ml) [26]. An in vivo study showed that daily topical application of 30 mg/ml EGCG (655 times higher than 100 μM) for 30 days failed to induce dermal toxicity [27]. In addition, the viability of the keratinocytes was confirmed by BrdU incorporation and SDH activity upon EGCG or GTPP-exposure, and their apoptotic status investigated by a caspase 3 activity assay; there was no major alteration in these measurements (Fig. 4). In order to assess whether increasing polyphenol concentrations themselves alter and/or interfere with the BrdU and MTT assays, an oral carcinoma cell line, OSC2, was treated identically. Both BrdU incorporation and MTT

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levels decreased significantly [4]. This result suggests that the effect of EGCG or GTPPs on exponentially growing keratinocytes is a selective induction of differentiation, in contrast to the apoptotic cell death initiated in OSC2 tumor cells.

Thus, we report here, for the first time that, at certain concentrations, EGCG or a mixture of the major green tea polyphenols stimulated aged keratinocytes to generate biological energy and to synthesize DNA, possibly for renewed cell division. For keratinocytes in an exponential growth phase, EGCG or a mixture of the major green tea polyphenols potently stimulated these cells to commit to differentiation with minimal impact on DNA synthesis or energy levels. Stimulating differentiation of keratinocytes in the basal layer of the epidermis and energizing and stimulating cell division/DNA synthesis in aged keratinocytes could potentially reduce the time of healing and prevent the formation of scar tissue, which occupies the space not repopulated by keratinocytes.

Therefore, green tea components may be useful topically for promoting skin regeneration, wound healing or treatment of certain epithelial conditions such as aphthous ulcers, psoriasis and actinic keratosis. In addition, the differentiation-inducing potential of green tea components might be beneficial to patients who have conditions characterized by abnormally accelerated skin cell growth and lack of differentiation. However, future studies are needed to determine the optimal time of treatment, concentrations and combinations of green tea polyphenols to be used topically for therapeutic intervention for various epithelial conditions.

FIGURE LEGENDS

Figure 1. EGCG and GTPPs Stimulate Mitochondrial Energy Production and DNA Synthesis in Aged Keratinocytes. Panels A, C and E. MTT assay results of normal human

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primary epidermal keratinocytes cultured for 15 days, 20 days or 25 days in KGM-2 medium, respectively, and treated with increasing concentrations of EGCG as indicated, or 0.2 mg/ml GTPPs for 24 h. Data represent the average and standard deviation of triplicate samples. Experiments were repeated five times with consistent results. Panels B, D and F. BrdU assay results of normal human primary epidermal keratinocytes cultured for 15 days, 20 days and 25 days in KGM-2 medium, respectively, and treated with increasing concentrations of EGCG as indicated or 0.2 mg/ml GTP for 24 h. Data represent the average and standard deviation of triplicate samples. Experiments were repeated three times with consistent results, and the above experiments were performed in parallel.

Figure 2. EGCG and GTPPs Stimulate Transglutaminase Activity in Exponentially Growing Keratinocytes. A. Pre-confluent, exponentially growing normal human primary epidermal keratinocytes without treatment. B. Pre-confluent normal human primary epidermal keratinocytes treated with 50 μM EGCG for 24 h. Morphological changes are evident, which suggests the cells have committed to differentiation. C. Comparison of transglutaminase activity (a late differentiation marker) between control and EGCG-treated cells. Cells treated with 50 μM or 100 μM EGCG have significantly higher activity. Data represent the average and standard deviation of triplicate samples. Experiments were repeated three times with similar results.

Figure 3. EGCG and GTPPs Increase Levels of Differentiation Markers in Exponentially Growing Keratinocytes. Panels A, C and E. Untreated cells. B. Cells treated with 100 μM EGCG for 24 h and stained with antibody specific for human p57. D. Cells treated with 100 μM EGCG and stained with antibody specific for human keratin 1. F. Cells treated with 100 μM EGCG and stained with antibody specific for human filaggrin. In all panels EGCG was shown to induce changes in cell morphology, increasing the number of enlarged, flattened, squame-like

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keratinocytes observed in the cultures. These cell characteristics are typical of differentiated keratinocytes, as indicated also by the enhanced expression of differentiation markers in these squamous cells. Original magnification: 400X. Experiments were repeated twice with similar results.

Figure 4. EGCG and GTPPs Exert Minimal Effects on DNA Synthesis and Do Not Alter Mitochondrial Energy Production or Apoptosis in Exponentially Growing Keratinocytes. Exponentially growing normal human primary epidermal keratinocytes were evaluated for DNA synthesis, caspase 3 activities and SDH activities following treatment with increasing concentrations of EGCG as indicated or 0.2 mg/ml GTP. The results of the BrdU assay showed a slight increase of BrdU incorporation (A), while the caspase 3 assay (B) and MTT assay (C) were not significantly affected. Data represent the average with SD of triplicate samples. All experiments were performed three times with similar results.

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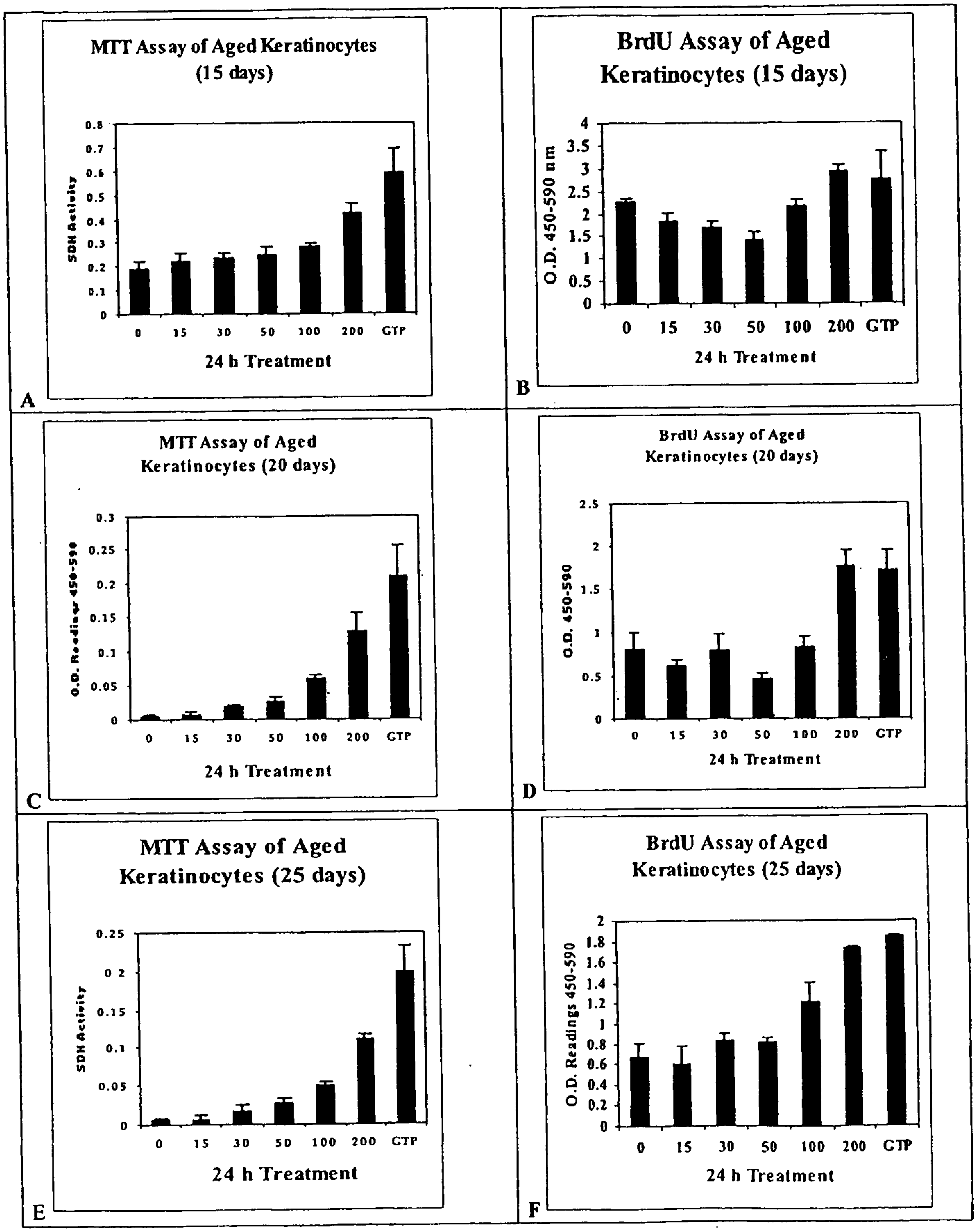
Figure 1


MTT Assay of Aged Keratinocytes (15 days)
SDH Activity
0.8
0.7
0.6
0.5
0.4
0.3
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15
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200
GTP
24 h Treatment
A
BrdU Assay of Aged Keratinocytes (15 days)
O.D. 450-590 nm
4
3.5
3
2.5
2
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0.5
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0
15
30
50
100
200
GTP
24 h Treatment
B
MTT Assay of Aged Keratinocytes (20 days)
O.D. Reading 450-590
0.3
0.25
0.2
0.15
0.1
0.05
0
0
15
30
50
100
200
GTP
24 h Treatment
C
BrdU Assay of Aged Keratinocytes (20 days)
O.D. 450-590
2.5
2
1.5
1
0.5
0
0
15
30
50
100
200
GTP
24 h Treatment
D
MTT Assay of Aged Keratinocytes (25 days)
SDH Activity
0.25
0.2
0.15
0.1
0.05
0
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15
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100
200
GTP
24 h Treatment
E
BrdU Assay of Aged Keratinocytes (25 days)
O.D. Readings 450-590
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1.4
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GTP
24 h Treatment
F

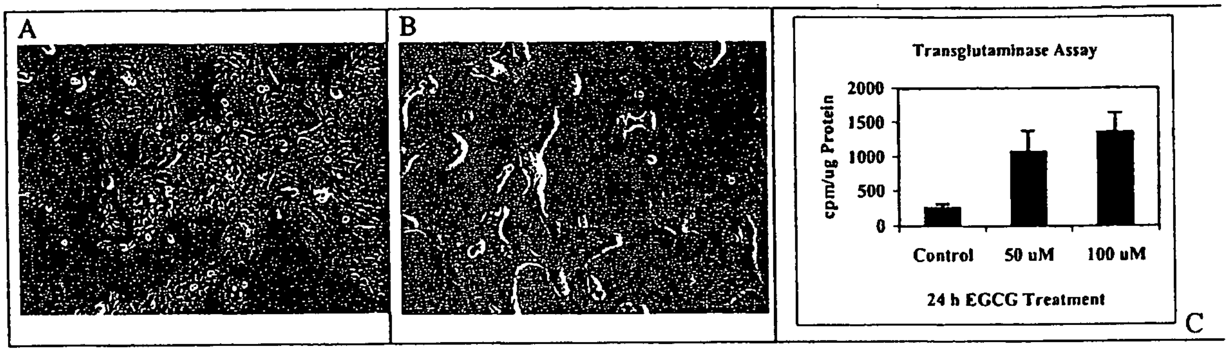


Figure 2

[redacted]

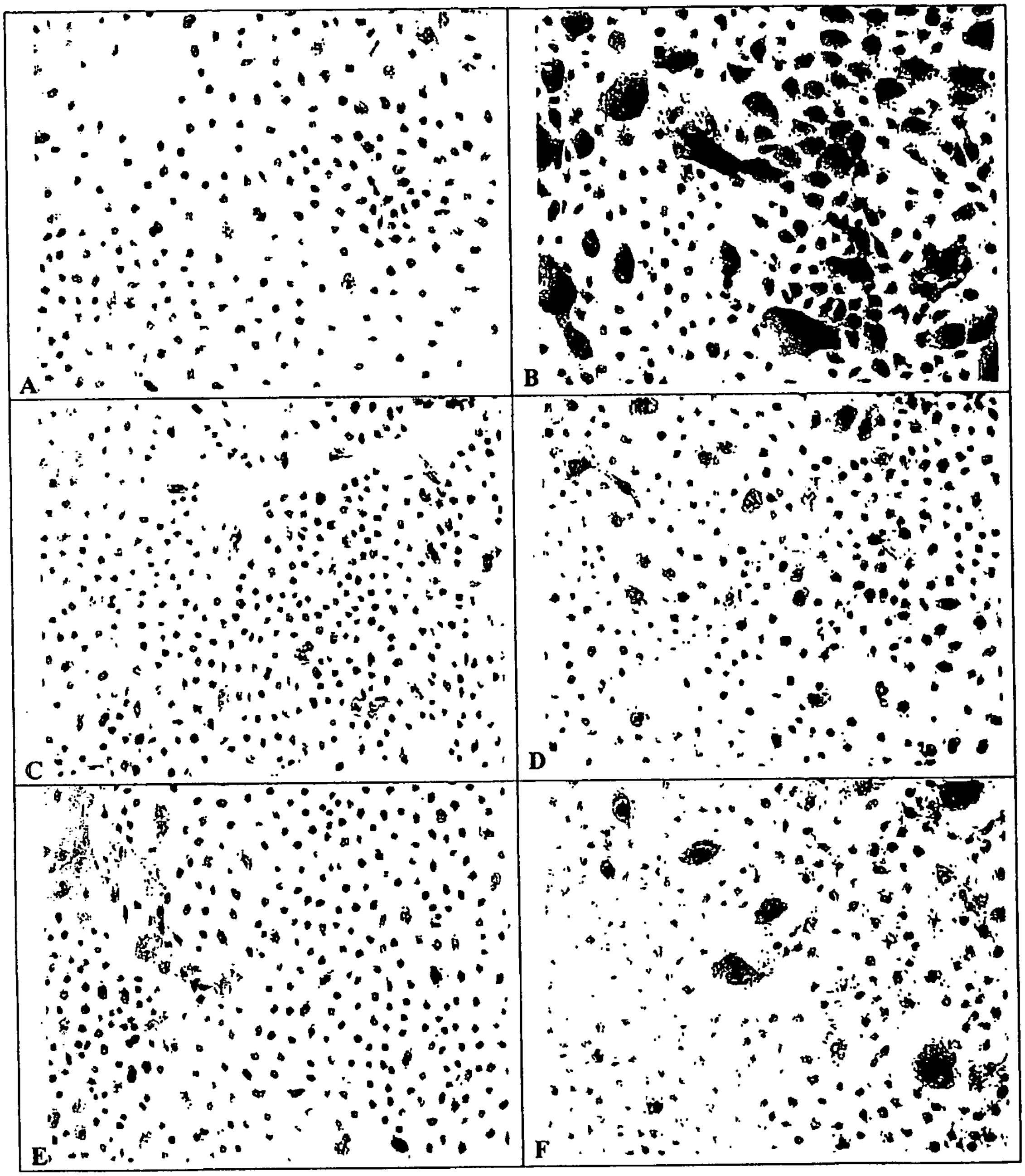
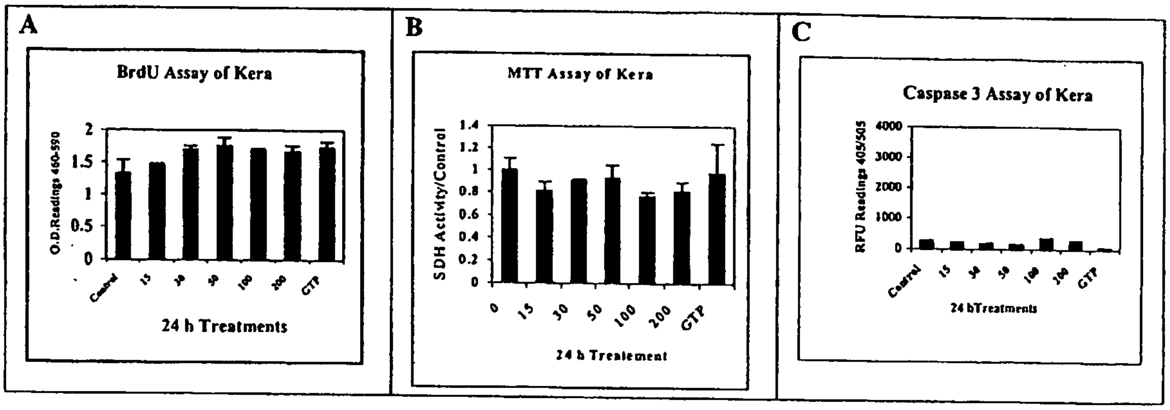


Figure 3


A
BrdU Assay of Kera
O.D.Readings 460-590
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1.5
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Control
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GTP
24 h Treatments
B
MTT Assay of Kera
SDH Activity/Control
1.4
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GTP
24 h Trealement
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Caspase 3 Assay of Kera
RFU Readings 405/505
4000
3000
2000
1000
0
Control
15
30
50
100
200
GTP
24 hTreatments

Figure 4

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The complete disclosures of all patents, patent applications including provisional patent applications, and publications, and electronically available material (e.g., GenBank amino acid and nucleotide sequence submissions) cited herein are incorporated by reference. The foregoing detailed description and examples have been provided for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described; many variations will be apparent to one skilled in the art and are intended to be included within the invention defined by the claims.

What is claimed is

1. A method of determining if cancer cells are resistant to an agent comprising:

determining the p57/KIP2 level in the cancer cells prior to contact with the agent;

contacting the cancer cells with the agent;

determining the p57/KIP2 level in the cancer cells after contact with the agent;

comparing the p57/KIP2 level in the cancer cells after contact with the agent to the p57/KIP2 level in the cancer cells prior to contact with the agent;

wherein an increase in the p57/KIP2 level in the cancer cells after contact with the agent compared to the p57/KIP2 level in the cancer cells prior to contact with the agent indicates the cancer cells are resistant to the agent.

2. A method of determining if cancer cells are resistant to an agent comprising:

contacting the cancer cells with the agent;

determining the p57/KIP2 level in the cancer cells after contact with the agent;

wherein an increase in the p57/KIP2 level in the cancer cells after contact with the agent indicates the cancer cells are resistant to the agent.

3. A method of determining if cancer cells are sensitive to an agent comprising:

determining the p57/KIP2 level in the cancer cells prior to contact with the agent;

contacting the cancer cells with the agent;

determining the p57/KIP2 level in the cancer cells after contact with the agent;

comparing the p57/KIP2 level in the cancer cells after contact with the agent to the p57/KIP2 level in the cancer cells prior to contact with the agent;

[redacted]

wherein no increase in the p57/KIP2 level in the cancer cells after contact with the agent compared to the p57/KIP2 levels in the cancer cells prior to contact with the agent indicates the cancer cells are sensitive to the agent.

4. A method of determining if cancer cells are sensitive to an agent comprising:

contacting the cancer cells with the agent;

determining the p57/KIP2 level in the cancer cells after contact with the agent;

wherein no increase in the p57/KIP2 level in the cancer cells after contact with the agent indicates the cancer cells are sensitive to the agent.

5. A method of identifying an agent effective for the treatment of a cancer comprising;

determining the p57/KIP2 level in cancer cells prior to contacting with the agent;

contacting the cancer cells with the agent;

determining the p57/KIP2 level in the cancer cells after contacting with the agent;

comparing the p57/KIP2 level in the cancer cells after contacting with the agent to the p57/KIP2 level in the cancer cells prior to contacting with the agent;

wherein no increase in the p57/KIP2 level in the cancer cells after contacting with the agent compared to the p57/KIP2 level in the cancer cells prior to contacting with the agent indicates the agent is effective for the treatment of a cancer.

6. A method of determining the therapeutic effectiveness of an agent comprising:

contacting normal cells with the agent;

determining the p57/KIP2 level in the normal cells after contacting with the agent;

contacting cancer cells with the agent;

determining the p57/KIP2 level in the cancer cells after contacting with the agent;

comparing the p57/KIP2 level in the normal cells after contacting with the agent to the p57/KIP2 level in the cancer cells after contacting with the agent;

wherein a higher p57/KIP2 level in the normal cells compared to the p57/KIP2 level in the cancer cells indicates the agent is effective for the treatment of cancer.

7. The method of claim 6, wherein the normal cells and cancer cells are cultured together.

8. A method of optimizing the formulation of an agent for the treatment of a cancer comprising:

contacting cancer cells with a first formulation of the agent;

determining the p57/KIP2 level in the cancer cells contacted with the first formulation of the agent;

contacting cancer cells with a second formulation of the agent;

determining the p57/KIP2 level in the cancer cells contacted with the second formulation of the agent;

comparing the p57/KIP2 level in the cancer cells contacted with the first formulation of the agent to the p57/KIP2 level in the cancer cells contacted with the second formulation of the agent;

wherein the formulation with the lower level of p57/KIP2 indicates the formulation of the agent more effective for the treatment of a cancer.

9. The method of any one claims 1-8, wherein the cancer cell is an epithelial carcinoma cell line.

[redacted]

10. The method of claim 9, wherein the epithelial carcinoma cell lines is selected from the group consisting of an oral squamous carcinoma cell line, a metastatic oral carcinoma cell line, and a breast epithelial carcinoma cell line.

11. The method of any one of claims 1-8, wherein the cancer cell is derived from a human epithelial carcinoma.

12. The method of claim 11, wherein the human epithelial carcinoma is selected from the group consisting of an oral squamous carcinoma, a metastatic oral carcinoma, and a breast epithelial carcinoma.

13. The method of any one of claims 1-12, wherein determining the p57/KIP2 level is by detecting the p57/KIP2 protein.

14. The method of any one of claims 1-12, wherein determining the p57/KIP2 level is by detecting the mRNA encoding p57/KIP2.

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